2010

Use of a 3D perfusion bioreactor with osteoblasts and osteoblast/endothelial cell co-cultures to improve tissue-engineered bone

Matthew J. Barron
Michigan Technological University

The Use of a 3D Perfusion Bioreactor with Osteoblasts and Osteoblast/Endothelial Cell Co-cultures to Improve Tissue-Engineered Bone

By
MATTHEW J. BARRON

A DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
(Biomedical Engineering)

MICHIGAN TECHNOLOGICAL UNIVERSITY
2010

This dissertation, "The Use of a 3D Perfusion Bioreactor with Osteoblasts and Osteoblast/Endothelial Cell Co-cultures to Improve Tissue-Engineered Bone," is hereby approved in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the field of Biomedical Engineering.

DEPARTMENT:
Biomedical Engineering

Signatures:

Dissertation Advisor _____

Seth Donahue, PhD **Date**

Co-Advisor _____

Jeremy Goldman, PhD **Date**

Department Chair _____

Sean Kirkpatrick, PhD **Date**

TABLE OF CONTENTS

List of Figures

List of Tables

Preface

This dissertation describes the effects of perfusion flow and mechanical stimulation in osteoblasts and osteoblast/endothelial cell co-cultures seeded in two-dimensions and on three-dimensional scaffolds for use in tissue engineering. It explores the use of a novel bioreactor for manipulating the mechanical environment of 3D cultures to maximize osteogenic and angiogenic potential. Several chapters in this dissertation are in preparation for publication and/or already in print in scientific journals.

- Chapter 1 is an introduction with sufficient background and rationale for the research proposed and performed, as well as the hypotheses and specific aims for each research project.

- Chapter 2 covers data generated while studying the effects of partitioning a mechanical stimulus into bouts with rest periods inserted. This data did not show significance, but represents the starting point for additional research comparing mechanical stimulation in 2D vs. 3D and perfusion flow vs. static culture in 3D.

- Chapter 3 describes the effects of mechanical stimulation on osteoblast cultures in 2D and 3D and compares their gene expression profiles. This manuscript is published in the Journal of Biomechanical Engineering (J Biomech Eng. 2010 Apr;132(4):041005). I performed the research and was the main author for this publication.

- Chapter 4 describes the effects of perfusion flow on 3D osteoblast cultures. This study determined gene expression for several osteogenic and angiogenic genes under varying treatments. This manuscript is being prepared for submission. I performed the research and was the main author for this publication.

- Chapter 5 covers data generated while running preliminary co-culture studies. Experiments determined an appropriate ratio of endothelial cells to use in conjunction with osteoblasts and also determined the effects of co-culture on osteoblast-specific gene expression.

- Chapter 6 investigated the pre-vascularization of 3D osteoblast cultures using endothelial cells. This study utilized osteoblast/endothelial cell co-cultures in a 3D perfusion bioreactor, exploring the effects of perfusion flow vs. static incubation. This manuscript is being prepared for submission. I performed the research and was the main author for this publication.

- Chapter 7 is a summary of all conclusions reached in this dissertation work.

ABSTRACT

The delivery of oxygen, nutrients, and the removal of waste are essential for cellular survival. Culture systems for 3D bone tissue engineering have addressed this issue by utilizing perfusion flow bioreactors that stimulate osteogenic activity through the delivery of oxygen and nutrients by low-shear fluid flow. It is also well established that bone responds to mechanical stimulation, but may desensitize under continuous loading. While perfusion flow and mechanical stimulation are used to increase cellular survival in vitro, 3D tissue-engineered constructs face additional limitations upon in vivo implantation. As it requires significant amounts of time for vascular infiltration by the host, implants are subject to an increased risk of necrosis. One solution is to introduce tissue-engineered bone that has been pre-vascularized through the co-culture of osteoblasts and endothelial cells on 3D constructs.

It is unclear from previous studies: **1**) how 3D bone tissue constructs will respond to partitioned mechanical stimulation, **2**) how gene expression compares in 2D and in 3D, **3**) how co-cultures will affect osteoblast activity, and **4**) how perfusion flow will affect co-cultures of osteoblasts and endothelial cells. We have used an integrated approach to address these questions by utilizing mechanical stimulation, perfusion flow, and a co-culture technique to increase the success of 3D bone tissue engineering. We measured gene expression of several osteogenic and angiogenic genes in both 2D and 3D (static culture and mechanical stimulation), as well as in 3D cultures subjected to perfusion flow, mechanical stimulation and partitioned mechanical stimulation. Finally, we co-cultured osteoblasts and endothelial cells on 3D scaffolds and subjected them to long-term incubation in either static culture or under perfusion flow to determine changes in gene expression as well as histological measures of osteogenic and angiogenic activity.

We discovered that 2D and 3D osteoblast cultures react differently to shear stress, and that partitioning mechanical stimulation does not affect gene expression in our model. Furthermore, our results suggest that perfusion flow may rescue 3D tissue-engineered constructs from hypoxic-like conditions by reducing hypoxia-specific gene expression and increasing histological indices of both osteogenic and angiogenic activity.

Future research to elucidate the mechanisms behind these results may contribute to a more mature bone-like structure that integrates more quickly into host tissue, increasing the potential of bone tissue engineering.

CHAPTER ONE
Introduction

Significance

There are over 1 million procedures that require the use of bone grafting each year in the United States. Bone transplants are sometimes necessary for spinal fusion, fracture healing, and defects resulting from trauma or disease (1-3). As the population continues to age, the prevalence of degenerative disk disease and incidence of osteoporosis will also increase, amplifying the incidence of bone grafting. Thus, the billion dollar market for bone graft substitutes (BGS) will continue to rise. Currently, approximately 40% of all grafting procedures utilize an autograft, which is harvested bone from a patient's own body. However, while autografts are currently the best available option for bone grafting, the pain and morbidity associated with second surgeries combined with the limited availability of autograft sources highlights the need for alternative grafting materials (1-3).

Several options are currently available as bone graft alternatives, including allografts (donor bone), demineralized bone matrix (crushed allograft with mineral removed), synthetic materials (polymeric or metallic scaffolds), growth factors (primarily bone morphogenetic protein or BMP), and orthopedic stem cell products (OSCP – stem cells combined with carrier). Ideally, any bone graft will; 1) provide scaffolding for new bone tissue to grow into (osteoconductive), 2) induce host tissue to produce new bone tissue (osteoinductive), and 3) supply a source for new tissue growth through osteoblasts or mesenchymal stem cells (osteogenic) (3-6). Of all bone graft alternatives, only orthopedic stem cell products provide all three essential traits.

Three-dimensional (3D) bone tissue engineering embodies the OSCP category. Bone tissue engineering is based on the use of a cell-seeded synthetic scaffold as a grafting substitute. Typically, the construct is cultured outside of the body (*in vitro*) for a period of time, allowing bone matrix to form within the porous scaffold. Upon *in vivo* implantation, a successfully engineered bone graft will be accepted by the host tissue, provide mechanical stability, and allow for remodeling of the construct by the host tissue

(1-3). While this approach is promising to be an effective therapy for skeletal defect repair, one of the biggest limitations to overcome is the in vitro maintenance of cellular viability within 3D constructs and in vivo vascular infiltration upon implantation.

Perfusion bioreactors

The delivery of oxygen, nutrients and the removal of waste are essential for cellular survival in vivo and in vitro. Because of its poor diffusion capacity, oxygen transport largely governs the production of 3D tissue cultures in vitro (7,8). In general, diffusion of oxygen and nutrients is restricted to about 200 μm, which is approximately the maximum distance between cells and capillaries in vivo (excepting cartilage) (4,7,9,10). As 3D in vitro tissue cultures lack oxygen- and nutrient-delivering vasculature, they suffer from inadequate mass transport, limiting the culture of viable 3D tissue (4,10).

The emergence of bioreactor technology has mitigated the transport limitations of in vitro 3D bone culture. Many types of bioreactors have been developed, with most falling into one of the following categories: rotating wall vessels, spinner flasks, or perfusion flow systems. Each has its benefits and limitations. Spinner flasks have the benefits of uniform cell seeding and center construct viability, but are limited in effect by the formation of outer fibrous capsules due to high levels of fluid shear stresses. Rotating wall vessels mitigate fibrous capsule formation by utilizing low magnitude fluid shear, but also subject cells to microgravity conditions which are not physiologically relevant. Of the three types of bioreactor, perhaps the most prevalent and broadest in scope is the perfusion bioreactor. Utilizing fluid flow to deliver culture medium to the center of cellular constructs, perfusion bioreactors have been modified to subject 3D cell-seeded scaffolds to a wide range of shear stresses under a variety of waveforms. In addition to being very adaptable, perfusion bioreactors have also been successfully used to create commercially viable engineered tissue (11).

Perfusion bioreactors deliver culture medium to cell-seeded porous scaffolds with the primary goal of improving chemotransport. Perfusion flow increases cellular infiltration and viability within 3D scaffolds and increases the level of oxygen transport (7,12-16). This leads to up-regulation in gene expression (12,14,17), increases in protein

production (13,16,18-21), and enhanced calcium deposition (13,19,22-24) in osteoblast-seeded scaffolds. Taken together, it is clear that there is great potential for the use perfusion bioreactors in 3D bone tissue engineering.

In general, perfusion bioreactors utilize low rates of fluid flow, maintaining fluid shear stresses much lower than physiological levels associated with mechanical loading (25). However, studies also show that increasing perfusion flow rate up-regulates osteogenic gene expression and matrix production, suggesting higher quality tissue formation. For example, osteopontin expression was directly correlated to the magnitude of perfusion flow in marrow stromal osteoblasts (13), PGE2 production was enhanced in osteoblasts by oscillatory shear stress (14,18), and mineralized matrix production increased with increasing shear (13,19). These 3D studies highlight the importance of a mechanically-regulated *in vitro* environment.

Effects of mechanical stimulation on bone

It is well established that bone responds to its mechanical environment both *in vivo* and *in vitro* (18,26-30). *In vivo*, mechanical loading increases bone volume (31), bone strength (30,32), and bone formation rate (33). Furthermore, *in vivo* loading increases the gene expression of vital matrix proteins such as osteocalcin (OCN), collagen 1 (Col 1), and alkaline phosphatase (ALP) (34,35). In general, excessive mechanical loading causes bone formation, and reduced loading activates bone resorption.

The adaptation of bone to mechanical loading is attributed to mechanotransduction, which is the conversion of mechanical stimuli into biochemical responses. However, the mechanisms behind this conversion are unclear. Electric fields, matrix strains, pressure gradients, and fluid shear stresses have all been considered as possible mechanical stimuli responsible for bone adaptation (26). One of the most popular theories suggests that as strains are imposed on bone, extracellular fluid is forced through the network of canaliculi and lacunae, acting as the primary stimulus behind osteocyte mechanosensation (36,37). Thus, fluid flow may serve as the prominent mediator in bone modeling, remodeling, and repair.

Historically, *in vitro* cultures have played a key role in the study of bone mechanobiology, with fluid flow remaining one of the most well studied stimuli. The vast majority of *in vitro* work has been done in two dimensions (2D), where magnitude, waveform, and rate of shear stress have proven to be important determinants of mechanically-induced gene expression in bone cells (38-43). Steady high shear stresses of 1-2 Pa are common in 2D studies, as predictions suggest these values to be within the range seen by bone cells *in vivo* under conditions of mechanical loading (25) . This high-shear fluid flow enhances osteogenic activity through the mediation of proliferation, differentiation, and matrix deposition (39,44-49).

One limitation with *in vitro* models is that 2D experiments are performed on flat surfaces, which do not mimic in vivo 3D architecture. As a result, 2D *in vitro* models do not provide physiological environments for mechanotransduction studies. Bone cells have been shown to react differently in 2D and 3D environments (50-53). For example, measures of both osteogenic and angiogenic activity were up-regulated in 3D cultures of bone cells compared to those cultured in 2D (54-56). Considering this, it is clear that 2D models do not always accurately represent what will occur in 3D conditions. Mechanical stimulation in 3D osteoblast cultures has not been studied extensively. However, there is literature showing that perfusion flow increases matrix production (12,18,56), mineralization (13,19,57), and late stage osteogenic gene expression (12,14) in osteoblastic cells cultured on 3D scaffolds. Since the perfusion flow utilized in the these studies resulted in shear stresses well below those experienced in vivo (25), there remains little data on the effects of physiologically-relevant mechanical stimulation in 3D (18,58). Thus, one of the aims of this dissertation work is to determine if flow-induced shear stress mediates gene expression similarly in osteoblast-like cells cultured in 2D and 3D environments.

While it is clear that mechanical stimulation affects bone both *in vivo* and *in vitro*, there is evidence that osteoblasts subjected to continuous mechanical stimulation may contain mechanisms that desensitize them to further loading after a certain period (32,59-62). *In vivo* work has shown that bone cells will accommodate to loading conditions after time, rendering continued loading ineffective (61). However, research has also shown that mechanosensitivity can be regained with rest; rat tibias responded more

osteogenically to shorter, partitioned bouts of mechanical stimulus as compared to a constant, prolonged bout (32). *In vitro* work has confirmed these studies, demonstrating that rest periods between bouts of mechanical stimulus allow osteoblastic cells to regain their mechanosensitivity (21,42,62). Taken together, these results suggest that it is possible to manipulate the mechanical environment of cells to produce a desired response, allowing scientists to maximize osteogenic potential. Therefore, the aims of this dissertation work include the study of partitioned mechanical stimulation in 3D cultures of osteoblast-like cells. This not only has direct clinical implications, but also presents *in vitro* opportunities to condense the culture time needed to produce high-quality tissue-engineered bone.

Vascularization of Bone

Over 200 years ago, Hunter predicted the importance of blood vessels to bone development and fracture repair (63,64). Since then, many studies have shown that proper vascularization is critical for successful bone growth and fracture healing (36,63,64). The vasculature provides bone tissue with oxygen, nutrients, and growth factors, as well as the removal of metabolic waste products. Without appropriate vascularization, bone tissue would face limited growth and hypoxia (64), decreased bone formation (65), and improper fracture healing (66).

As bone tissue-engineering is emerging as a potential source of tissue implants, vascularization must be considered as it is critical for graft acceptance (67-72). One of the major limitations of tissue-engineered bone is that once implanted *in vivo*, significant amounts of time are required for vascular integration. Current methods rely on the in-growth of vasculature from host tissue (68,70), which can lead to poor vascularization and implant rejection (68). One solution to this clinical problem is to introduce tissue-engineered bone that has been pre-vascularized by a rudimentary vascular system (68,70,71,73,74). Approaches to this solution include the co-culture of bone cells and endothelial cells on three-dimensional constructs.

Endothelial cells are major constituents of the vasculature that function as a barrier and are capable of direct communication with adjacent tissue and circulating blood cells (68,69,75-77). Bone is a highly vascularized tissue; haversian canals in cortical bone and

marrow spaces in trabecular bone are lined with capillary-rich endothelium. As haversian canals and trabeculae also contain bone lining cells (quiescent osteoblasts), the intimate relationship between osteoblasts and endothelial cells is apparent.

It was once thought that endothelial cells were simply conduits for the transport of nutrients and waste (75,78). Now, it is recognized that endothelial cells are actively involved in bone growth, development, and maintenance. Moreover, communication between the bone and vasculature is necessary for healthy bone, and angiogenesis and osteogenesis are considered to be tightly coupled processes (63). Endothelial cells produce and secrete a number of mediators (e.g. NO, FGF, prostaglandins) that contribute to the control of osteoblast activity. Likewise, osteoblasts synthesize an array of cytokines and growth factors (most notably vascular endothelial growth factor) that influence endothelial cells (63,77,79).

There has been some success with co-culturing bone cells and endothelial cells to overcome the lack of vasculature in tissue-engineered bone (68-71,73,74,80). These studies have shown that not only will endothelial cells form a pre-vascular network during co- culture (70,71,73), but also that endothelial cells can augment osteoblastic activity when in direct contact with osteoblasts (69,70,73,77,81). Furthermore, recent work has shown the *in vivo* integration of 3D co-cultures, with the formation of an improved vascular network upon implantation (72,82-84).

While it is known that endothelial cells and osteoblasts share a positive synergy in co-culture, many early systems utilized either static 2D cultures or a spheroidal 3D pellet culture model that would not sustain mechanical loading as an implant, nor serve as an osteoconductive scaffold for host integration. There is little data on the response of co-cultures on a porous 3D scaffold, and less data on how a dynamic environment will affect them. Therefore, the aims of this dissertation work include the study of osteoblast and endothelial cell co-cultures on 3D scaffolds in a perfusion bioreactor.

Rationale, Hypotheses, and Specific Aims for Dissertation Research

Mechanical Stimulation in 3D

2D fluid flow studies have yielded an abundance of data clarifying cellular signaling mechanisms following mechanical stimulation. Stimulation causes bone cells to up-regulate NO, OPN, PGE2, and COX-2 (48,49,85-87), and the signaling pathways responsible for these changes have also been studied (41,48,88) . While 2D studies are valuable, they do not represent a physiologically-relevant model for mechanotransduction. Since bone cells have been shown to react differently in 2D and 3D environments (50-53), it is important to consider 3D models as responses to mechanical stimulation may be different. Although perfusion bioreactors are widely used to culture bone in 3D, these studies use flow that is orders of magnitude lower than what is considered to be mechanical stimulation (12,13,19,23,57,89). Additionally, most studies are interested in long term responses to perfusion flow, such as matrix production and calcium deposition. Thus, there remains a paucity of data on early responses to perfusion flow and the effects of mechanical stimulation on 3D cultures. Furthermore, while partitioned loading schemes have been tested both in vivo and in 2D in vitro studies (32,46,47,62), it is not known how 3D cultures will respond. Therefore, the goals of this study were: 1) determine if flow-induced shear stress mediates gene expression similarly in osteoblast-like cells cultured in 2D and 3D environments, and 2) determine whether subjecting 3D cultures to partitioned mechanical stimulation will provoke a distinct osteogenic response as compared to continuous stimulation, perfusion flow or static culture.

Hypothesis I: Osteoblasts cultured in 3D will up-regulate mRNA levels of osteogenic-specific genes when subjected to partitioned mechanical stimulation as compared to mechanical stimulation, perfusion flow, or static culture.

Specific Aim – Determine gene expression for osteogenic and angiogenic activity in 2D and 3D cultures subjected to mechanical stimulation, and in 3D cultures under static, perfusion, mechanical, and partitioned mechanical treatments.

Co-culture in 2D and 3D

The ultimate goal of bone tissue engineering is to produce usable bone in vitro that will remain viable upon implantation and fully integrate with the host tissue. Since it requires time for host vasculature to infiltrate implanted tissue, efforts have been made to circumvent this issue by pre-vascularizing tissue engineered bone (68,70,71,73,74). One approach is to co-culture bone and endothelial cells on 3D constructs prior to implantation with the hope that a rudimentary vascular-like network will form, accelerating vascular integration with host tissue. To date, there has been some success with co-culturing bone cells and endothelial cells to overcome the lack of vasculature in tissue-engineered bone (68-71,73,74,80). These studies have shown that not only will endothelial cells form a pre-vascular network during co- culture (70,71,73), but also that endothelial cells can augment osteoblastic activity when in direct contact with osteoblasts (69,70,73,77,81). However, most co-culture systems utilize a spheroidal culture model that would not sustain mechanical loading as an implant, nor serve as an osteoconductive scaffold for host integration. Since implants would experience a mechanical load in vivo, a 3D model that can withstand loads is desirable. It is not known whether an osteoblast/endothelial cell co-culture seeded onto a porous scaffold will change osteogenic or angiogenic gene expression compared to osteoblast cultures alone.

Hypothesis II: Osteoblast/endothelial cell co-cultures will result in higher expression of osteogenic and angiogenic genes as compared to osteoblasts cultured alone in 3D environments. Furthermore, endothelial cells will form a vascular-like network when in co-culture.

Specific Aim: Quantify gene expression of osteogenic and angiogenic activity over time. Real-time PCR will quantify mRNA levels of bone markers (type 1 collagen, osteopontin, osteocalcin, alkaline phosphatase, Cox-2) and angiogenic markers (VEGF and M-CSF). The ratio of endothelial cells to osteoblasts that produces the greatest increase in gene expression will also be determined.

Co-culture in a perfusion bioreactor

While co-cultures have proven to form vascular-like networks in addition to up-regulating osteogenic activity, there is no literature describing the dynamic culture of a co-culture system. Thus, it is not known how a co-culture model will respond to the dynamic environment provided by a perfusion bioreactor. Since it is well known that fluid flow activates both osteoblasts and endothelial cells (63,77,79,90), we propose that a co-culture model under perfusion flow will increase osteogenic and angiogenic gene expression. Furthermore, endothelial cells have also been known to form tube-like structures more quickly when exposed to fluid flow (91). So, in addition to increases in gene expression, we also expect to see an increase in tube-like formation in samples cultured under fluid flow. This work will help elucidate the contributions of endothelial cells to osteoblastic gene activity, as well as the effects that a dynamic environment has on the formation of a vascular network in co-cultures. This will be the first study of osteoblast and endothelial cell co-cultures in a perfusion bioreactor.

Hypothesis III: Osteoblast/endothelial cell co-cultures subjected to perfusion flow will increase osteogenic activity compared to static cultures. In addition, culture in the perfusion bioreactor will increase the length and number of endothelial tube-like formations.

Specific Aim: Quantify gene expression of osteogenic and angiogenic activity over time. Real-time PCR will quantify mRNA levels of VEGF, M-CSF, type 1 collagen, osteopontin, osteocalcin, alkaline phosphatase, and Cox-2. Osteoid deposition and vessel formation over time with and without perfusion flow will also be quantified.

References

1. Yaszemski MJ, Payne RG, Hayes WC, Langer R, Mikos AG 1996 Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. Biomaterials **17**(2)**:**175-85.

2. Bauer TW 2007 An overview of the histology of skeletal substitute materials. Arch Pathol Lab Med **131**(2)**:**217-24.

3. Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA 2007 Cell-Based Bone Tissue Engineering. PLoS Med **4**(2)**:**e9.

4. Muschler GF, Nakamoto C, Griffith LG 2004 Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am **86-A**(7)**:**1541-58.

5. Langer R, Vacanti JP, Vacanti CA, Atala A, Freed LE, Vunjak-Novakovic G 1995 Tissue engineering: biomedical applications. Tissue Eng **1**(2)**:**151-61.

6. Galletti PM, Hellman KB, Nerem RM 1995 Tissue engineering: from basic science to products: a preface. Tissue Eng **1**(2)**:**147-9.

7. Volkmer E, Drosse I, Otto S, Stangelmayer A, Stengele M, Kallukalam BC, Mutschler W, Schieker M 2008 Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. Tissue Eng Part A **14**(8)**:**1331-40.

8. Malda J, Klein TJ, Upton Z 2007 The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng **13**(9)**:**2153-62.

9. Arkudas A, Beier JP, Heidner K, Tjiawi J, Polykandriotis E, Srour S, Sturzl M, Horch RE, Kneser U 2007 Axial prevascularization of porous matrices using an arteriovenous loop promotes survival and differentiation of transplanted autologous osteoblasts. Tissue Eng **13**(7)**:**1549-60.

10. Griffith CK, Miller C, Sainson RC, Calvert JW, Jeon NL, Hughes CC, George SC 2005 Diffusion limits of an in vitro thick prevascularized tissue. Tissue Eng **11**(1-2)**:**257-66.

11. Chen HC, Hu YC 2006 Bioreactors for tissue engineering. Biotechnol Lett **28**(18)**:**1415-23.

12. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE 2003 Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng **9**(6)**:**1197- 203.

13. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG 2002 Fluid flow increases mineralized matrix deposition in 3D

perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A **99**(20)**:**12600-5.

14. Jaasma MJ, O'Brien FJ 2008 Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A **14**(7)**:**1213-23.

15. Du D, Furukawa KS, Ushida T 2009 3D culture of osteoblast-like cells by unidirectional or oscillatory flow for bone tissue engineering. Biotechnol Bioeng **102**(6)**:**1670-8.

16. Li D, Tang T, Lu J, Dai K 2009 Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. Tissue Eng Part A **15**(10)**:**2773-83.

17. Holtorf HL, Jansen JA, Mikos AG 2006 Modulation of cell differentiation in bone tissue engineering constructs cultured in a bioreactor. Adv Exp Med Biol **585:**225-41.

18. Vance J, Galley S, Liu DF, Donahue SW 2005 Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release. Tissue Eng **11**(11-12)**:**1832-9.

19. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG 2003 Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci U S A **100**(25)**:**14683-8.

20. Wang Y, Uemura T, Dong J, Kojima H, Tanaka J, Tateishi T 2003 Application of perfusion culture system improves in vitro and in vivo osteogenesis of bone marrow-derived osteoblastic cells in porous ceramic materials. Tissue Eng **9**(6)**:**1205-14.

21. Plunkett NA, Partap S, O'Brien FJ Osteoblast response to rest periods during bioreactor culture of collagen-glycosaminoglycan scaffolds. Tissue Eng Part A **16**(3)**:**943- 51.

22. Zhao F, Chella R, Ma T 2007 Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: Experiments and hydrodynamic modeling. Biotechnol Bioeng **96**(3)**:**584-95.

23. Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG 2006 In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci U S A **103**(8)**:**2488-93.

24. Holtorf HL, Datta N, Jansen JA, Mikos AG 2005 Scaffold mesh size affects the osteoblastic differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor. J Biomed Mater Res A **74**(2)**:**171-80.

25. Weinbaum S, Cowin SC, Zeng Y 1994 A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech **27**(3)**:**339-60.

26. Rubin J, Rubin C, Jacobs CR 2006 Molecular pathways mediating mechanical signaling in bone. Gene **367:**1-16.

27. Iqbal J, Zaidi M 2005 Molecular regulation of mechanotransduction. Biochem Biophys Res Commun **328**(3)**:**751-5.

28. Weiss S, Zimmermann G, Baumgart R, Kasten P, Bidlingmaier M, Henle P 2005 Systemic regulation of angiogenesis and matrix degradation in bone regeneration--distraction osteogenesis compared to rigid fracture healing. Bone **37**(6)**:**781-90.

29. Li G, Simpson AH, Kenwright J, Triffitt JT 1999 Effect of lengthening rate on angiogenesis during distraction osteogenesis. J Orthop Res **17**(3)**:**362-7.

30. Robling AG, Castillo AB, Turner CH 2006 Biomechanical and molecular regulation of bone remodeling. Annu Rev Biomed Eng **8:**455-98.

31. van der Meulen MC, Morgan TG, Yang X, Baldini TH, Myers ER, Wright TM, Bostrom MP 2006 Cancellous bone adaptation to in vivo loading in a rabbit model. Bone **38**(6)**:**871-7.

32. Robling AG, Burr DB, Turner CH 2000 Partitioning a daily mechanical stimulus into discrete loading bouts improves the osteogenic response to loading. J Bone Miner Res **15**(8)**:**1596-602.

33. Schriefer JL, Warden SJ, Saxon LK, Robling AG, Turner CH 2005 Cellular accommodation and the response of bone to mechanical loading. J Biomech **38**(9)**:**1838-45.

34. Moalli MR, Caldwell NJ, Patil PV, Goldstein SA 2000 An in vivo model for investigations of mechanical signal transduction in trabecular bone. J Bone Miner Res **15**(7)**:**1346-53.

35. Pavlin D, Zadro R, Gluhak-Heinrich J 2001 Temporal pattern of stimulation of osteoblast-associated genes during mechanically-induced osteogenesis in vivo: early responses of osteocalcin and type I collagen. Connect Tissue Res **42**(2)**:**135-48.

36. John P. Bilezikian LGR, Gideon A. Rodan 2002 Principles of Bone Biology. In: Bilezikian R, Rodan (ed.) Principles of Bone Biology, 2nd ed., vol. 1 and 2. Academic Press, San Diego, pp 1700.

37. Cowin SC, Moss-Salentijn L, Moss ML 1991 Candidates for the mechanosensory system in bone. J Biomech Eng **113**(2)**:**191-7.

38. Li YJ, Batra NN, You L, Meier SC, Coe IA, Yellowley CE, Jacobs CR 2004 Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. J Orthop Res **22**(6)**:**1283-9.

39. You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR 2001 Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem **276**(16)**:**13365-71.

40. Ponik SM, Triplett JW, Pavalko FM 2007 Osteoblasts and osteocytes respond differently to oscillatory and unidirectional fluid flow profiles. J Cell Biochem **100**(3)**:**794-807.

41. Wu CC, Li YS, Haga JH, Wang N, Lian IY, Su FC, Usami S, Chien S 2006 Roles of MAP kinases in the regulation of bone matrix gene expressions in human osteoblasts by oscillatory fluid flow. J Cell Biochem **98**(3)**:**632-41.

42. Batra NN, Li YJ, Yellowley CE, You L, Malone AM, Kim CH, Jacobs CR 2005 Effects of short-term recovery periods on fluid-induced signaling in osteoblastic cells. J Biomech **38**(9)**:**1909-17.

43. Sittichockechaiwut A, Scutt AM, Ryan AJ, Bonewald LF, Reilly GC 2009 Use of rapidly mineralising osteoblasts and short periods of mechanical loading to accelerate matrix maturation in 3D scaffolds. Bone **44**(5)**:**822-9.

44. Chen NX, Ryder KD, Pavalko FM, Turner CH, Burr DB, Qiu J, Duncan RL 2000 Ca(2+) regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts. Am J Physiol Cell Physiol **278**(5)**:**C989-97.

45. Donahue SW, Jacobs CR, Donahue HJ 2001 Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. Am J Physiol Cell Physiol **281**(5)**:**C1635-41.

46. Donahue TL, Haut TR, Yellowley CE, Donahue HJ, Jacobs CR 2003 Mechanosensitivity of bone cells to oscillating fluid flow induced shear stress may be modulated by chemotransport. J Biomech **36**(9)**:**1363-71.

47. Jacobs CR, Yellowley CE, Davis BR, Zhou Z, Cimbala JM, Donahue HJ 1998 Differential effect of steady versus oscillating flow on bone cells. J Biomech **31**(11)**:**969-76.

48. Wadhwa S, Choudhary S, Voznesensky M, Epstein M, Raisz L, Pilbeam C 2002 Fluid flow induces COX-2 expression in MC3T3-E1 osteoblasts via a PKA signaling pathway. Biochem Biophys Res Commun **297**(1)**:**46-51.

49. Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, Pilbeam CC 2002 Fluid flow induction of cyclo-oxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. J Bone Miner Res **17**(2)**:**266-74.

50. Liu H, Lin J, Roy K 2006 Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells. Biomaterials **27**(36)**:**5978-89.

51. Fischbach C, Kong HJ, Hsiong SX, Evangelista MB, Yuen W, Mooney DJ 2009 Cancer cell angiogenic capability is regulated by 3D culture and integrin engagement. Proc Natl Acad Sci U S A **106**(2)**:**399-404.

52. Byrne EM, Farrell E, McMahon LA, Haugh MG, O'Brien FJ, Campbell VA, Prendergast PJ, O'Connell BC 2008 Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation. J Mater Sci Mater Med **19**(11)**:**3455-63.

53. Farrell E, Byrne EM, Fischer J, O'Brien FJ, O'Connell BC, Prendergast PJ, Campbell VA 2007 A comparison of the osteogenic potential of adult rat mesenchymal stem cells cultured in 2-D and on 3-D collagen glycosaminoglycan scaffolds. Technol Health Care **15**(1)**:**19-31.

54. Hishikawa K, Miura S, Marumo T, Yoshioka H, Mori Y, Takato T, Fujita T 2004 Gene expression profile of human mesenchymal stem cells during osteogenesis in three-dimensional thermoreversible gelation polymer. Biochem Biophys Res Commun **317**(4)**:**1103-7.

55. Helmke C 1999 Factors affecting bone cell growth and differentiation under differing culture conditions, vol. Master of Arts. Rice University, Houston, pp 75.

56. Jarrahy R, Huang W, Rudkin GH, Lee JM, Ishida K, Berry MD, Sukkarieh M, Wu BM, Yamaguchi DT, Miller TA 2005 Osteogenic differentiation is inhibited and angiogenic expression is enhanced in MC3T3-E1 cells cultured on three-dimensional scaffolds. Am J Physiol Cell Physiol **289**(2)**:**C408-14.

57. Holtorf HL, Jansen JA, Mikos AG 2005 Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone. J Biomed Mater Res A **72**(3)**:**326-34.

58. Tanaka SM, Sun HB, Roeder RK, Burr DB, Turner CH, Yokota H 2005 Osteoblast responses one hour after load-induced fluid flow in a three-dimensional porous matrix. Calcif Tissue Int **76**(4)**:**261-71.

59. Robling AG, Burr DB, Turner CH 2001 Recovery periods restore mechanosensitivity to dynamically loaded bone. J Exp Biol **204**(Pt 19)**:**3389-99.

60. Robling AG, Turner CH 2002 Mechanotransduction in bone: genetic effects on mechanosensitivity in mice. Bone **31**(5)**:**562-9.

61. Saxon LK, Robling AG, Alam I, Turner CH 2005 Mechanosensitivity of the rat skeleton decreases after a long period of loading, but is improved with time off. Bone **36**(3)**:**454-64.

62. Donahue SW, Donahue HJ, Jacobs CR 2003 Osteoblastic cells have refractory periods for fluid-flow-induced intracellular calcium oscillations for short bouts of flow and display multiple low-magnitude oscillations during long-term flow. J Biomech **36**(1)**:**35-43.

63. Carano RA, Filvaroff EH 2003 Angiogenesis and bone repair. Drug Discov Today **8**(21)**:**980-9.

64. Glowacki J 1998 Angiogenesis in fracture repair. Clin Orthop Relat Res (355 Suppl)**:**S82-9.

65. Burkhardt R, Kettner G, Bohm W, Schmidmeier M, Schlag R, Frisch B, Mallmann B, Eisenmenger W, Gilg T 1987 Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. Bone **8**(3)**:**157-64.

66. Hausman MR, Schaffler MB, Majeska RJ 2001 Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone **29**(6)**:**560-4.

67. Kanczler JM, Oreffo RO 2008 Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater **15:**100-14.

68. Choong CS, Hutmacher DW, Triffitt JT 2006 Co-culture of Bone Marrow Fibroblasts and Endothelial Cells on Modified Polycaprolactone Substrates for Enhanced Potentials in Bone Tissue Engineering. Tissue Eng.

69. Stahl A, Wenger A, Weber H, Stark GB, Augustin HG, Finkenzeller G 2004 Bi-directional cell contact-dependent regulation of gene expression between endothelial cells and osteoblasts in a three-dimensional spheroidal coculture model. Biochem Biophys Res Commun **322**(2)**:**684-92.

70. Wenger A, Kowalewski N, Stahl A, Mehlhorn AT, Schmal H, Stark GB, Finkenzeller G 2005 Development and characterization of a spheroidal coculture model of endothelial cells and fibroblasts for improving angiogenesis in tissue engineering. Cells Tissues Organs **181**(2)**:**80-8.

71. Unger RE, Sartoris A, Peters K, Motta A, Migliaresi C, Kunkel M, Bulnheim U, Rychly J, Kirkpatrick CJ 2007 Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials. Biomaterials **28**(27)**:**3965-76.

72. Santos MI, Reis RL Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. Macromol Biosci **10**(1)**:**12-27.

73. Rouwkema J, de Boer J, Van Blitterswijk CA 2006 Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. Tissue Eng **12**(9)**:**2685-93.

74. Villars F, Guillotin B, Amedee T, Dutoya S, Bordenave L, Bareille R, Amedee J 2002 Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. Am J Physiol Cell Physiol **282**(4)**:**C775-85.

75. Streeten EA, Brandi ML 1990 Biology of bone endothelial cells. Bone Miner **10**(2)**:**85-94.

76. Brandi ML, Collin-Osdoby P 2006 Vascular biology and the skeleton. J Bone Miner Res **21**(2)**:**183-92.

77. Villars F, Bordenave L, Bareille R, Amedee J 2000 Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? J Cell Biochem **79**(4)**:**672-85.

78. Collin-Osdoby P 1994 Role of vascular endothelial cells in bone biology. J Cell Biochem **55**(3)**:**304-9.

79. Street J, Bao M, deGuzman L, Bunting S, Peale FV, Jr., Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van Bruggen N, Redmond HP, Carano RA, Filvaroff EH 2002 Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci U S A **99**(15)**:**9656-61.

80. Akita S, Tamai N, Myoui A, Nishikawa M, Kaito T, Takaoka K, Yoshikawa H 2004 Capillary vessel network integration by inserting a vascular pedicle

enhances bone formation in tissue-engineered bone using interconnected porous hydroxyapatite ceramics. Tissue Eng **10**(5-6)**:**789-95.

81. Clarkin CE, Emery RJ, Pitsillides AA, Wheeler-Jones CP 2008 Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated crosstalk to endothelial cells. J Cell Physiol **214**(2)**:**537-44.

82. Steffens L, Wenger A, Stark GB, Finkenzeller G 2009 In vivo engineering of a human vasculature for bone tissue engineering applications. J Cell Mol Med **13**(9B)**:**3380-6.

83. Yu H, VandeVord PJ, Mao L, Matthew HW, Wooley PH, Yang SY 2009 Improved tissue-engineered bone regeneration by endothelial cell mediated vascularization. Biomaterials **30**(4)**:**508-17.

84. Fidkowski C, Kaazempur-Mofrad MR, Borenstein J, Vacanti JP, Langer R, Wang Y 2005 Endothelialized microvasculature based on a biodegradable elastomer. Tissue Eng **11**(1-2)**:**302-9.

85. Bacabac RG, Smit TH, Mullender MG, Dijcks SJ, Van Loon JJ, Klein-Nulend J 2004 Nitric oxide production by bone cells is fluid shear stress rate dependent. Biochem Biophys Res Commun **315**(4)**:**823-9.

86. Mullender MG, Dijcks SJ, Bacabac RG, Semeins CM, Van Loon JJ, Klein-Nulend J 2006 Release of nitric oxide, but not prostaglandin E2, by bone cells depends on fluid flow frequency. J Orthop Res **24**(6)**:**1170-7.

87. Reich KM, Frangos JA 1991 Effect of flow on prostaglandin E2 and inositol trisphosphate levels in osteoblasts. Am J Physiol **261**(3 Pt 1)**:**C428-32.

88. Bakker AD, Klein-Nulend J, Burger EH 2003 Mechanotransduction in bone cells proceeds via activation of COX-2, but not COX-1. Biochem Biophys Res Commun **305**(3)**:**677-83.

89. Goldstein AS, Juarez TM, Helmke CD, Gustin MC, Mikos AG 2001 Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. Biomaterials **22**(11)**:**1279-88.

90. Ozawa N, Shichiri M, Iwashina M, Fukai N, Yoshimoto T, Hirata Y 2004 Laminar shear stress up-regulates inducible nitric oxide synthase in the endothelium. Hypertens Res **27**(2)**:**93-9.

91. Yamamoto K, Takahashi T, Asahara T, Ohura N, Sokabe T, Kamiya A, Ando J 2003 Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. J Appl Physiol **95**(5)**:**2081-8.

CHAPTER TWO
Partitioned Mechanical Stimulation

Introduction

Three-dimensional (3D) bone tissue engineering is promising to be an effective therapy for skeletal defect repair. However, diffusional constraints subject 3D cultures to limited cellular viability in the center of larger constructs (1,2). Consequently, researchers have developed perfusion bioreactors which deliver nutrients and oxygen to 3D scaffolds, as well as remove waste (1-5). Indeed, perfusion flow increases cellular viability within the center of 3D scaffolds (1,2). Although the use of perfusion flow is promising, high-shear flow rates have been shown to reduce cell number on 3D scaffolds due to increasing shear stress (1-3).

Fluid shear is recognized as a major mediator of osteogenic activity, and has potential to enhance bone tissue engineering procedures (2,6,7). Shear stress enhances osteogenic activity through the mediation of osteoblast proliferation, differentiation, matrix production, apoptosis, and angiogenic gene expression of bone cells. The magnitude, waveform, and rate of shear stress are important determinants of mechanically-induced gene expression in bone cells. Fluid flow studies involve loading regimes that include various flow types over several hours of stimulation. Steady shear stresses of 1-2 Pa are common, as predictions suggest these values to be within the range seen by bone cells in vivo (8) .

While mechanical stimulation can improve osteogenic activity, there is evidence that osteoblasts subjected to continuous mechanical stimulation may contain mechanisms that desensitize them to further loading after a certain period. Rat tibias loaded in four-point bending responded more osteogenically to shorter, partitioned bouts of mechanical stimulus as compared to less frequent, prolonged bouts (9). Furthermore, in vitro studies demonstrate that rest periods between bouts of mechanical stimulus allow osteoblastic cells to regain their mechanosensitivity (10,11).

3D perfusion cultures have recently provided valuable data concerning the disparate effects of varied shear rate on bone growth (1-4,12-15). Osteoblasts exposed to varying rates of perfusion (0.01-1.0 ml/min) had the greatest cellular viability and proliferation at the lowest flow rates (1). On the other hand, gene expression of several bone-specific proteins (OCN, OPN, ALP) increased as the flow rate increased. Taken together, this suggests that high rates of flow up-regulate gene expression, but reduce cellular viability when applied continuously.

In the present study, we propose to utilize both high and low rates of flow in a perfusion bioreactor to determine whether intermittent bouts of fluid shear can mediate growth-related, angiogenic, and apoptotic gene expression in osteoblasts seeded in 3D and exposed to continuous perfusion flow . Using low-shear perfusion flow to maintain cell-viability and high-shear flow as mechanical stimulation, we hypothesize that MC3T3 pre-osteoblast-seeded calcium phosphate scaffolds cultured in a 3D perfusion bioreactor will elicit a higher osteogenic response when exposed to intermittent mechanical loading as compared to a single bout of mechanical stimulation, perfusion flow alone, or static cultures.

Materials and Methods

Cell Culture

Mouse osteoblast-like cells (MC3T3-E1 subclone 14, ATCC, Manassas, VA) between passage numbers 20 and 26 were cultured in alpha-MEM (Invitrogen, Carlsband, CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Once cells reached ~70% confluency, they were trypsinized, re-suspended in culture medium, and statically seeded onto 3D calcium phosphate scaffolds (1,000,000 cells/scaffold) (BD Biosciences, San Jose, CA). The scaffolds have an interconnected porosity of ~ 60% with an average pore size of 200-400 microns. This is comparable to trabecular bone (50-90% porosity, 500-1500 micron pore size (16)), and within the range suggested to be optimal for bone regeneration (150 – 650 microns) in porous scaffolds (17). Samples were then incubated for 1 hour to allow cell adhesion, and were covered with media and incubated for 24 hours before experiments began.

Mechanical Loading in 3D

A custom perfusion bioreactor was used to mechanically stimulate osteoblasts on calcium phosphate scaffolds. Each bioreactor chamber consists of a top and bottom aluminum block, between which a polycarbonate block is secured (Fig. 1). A 6-mm hole

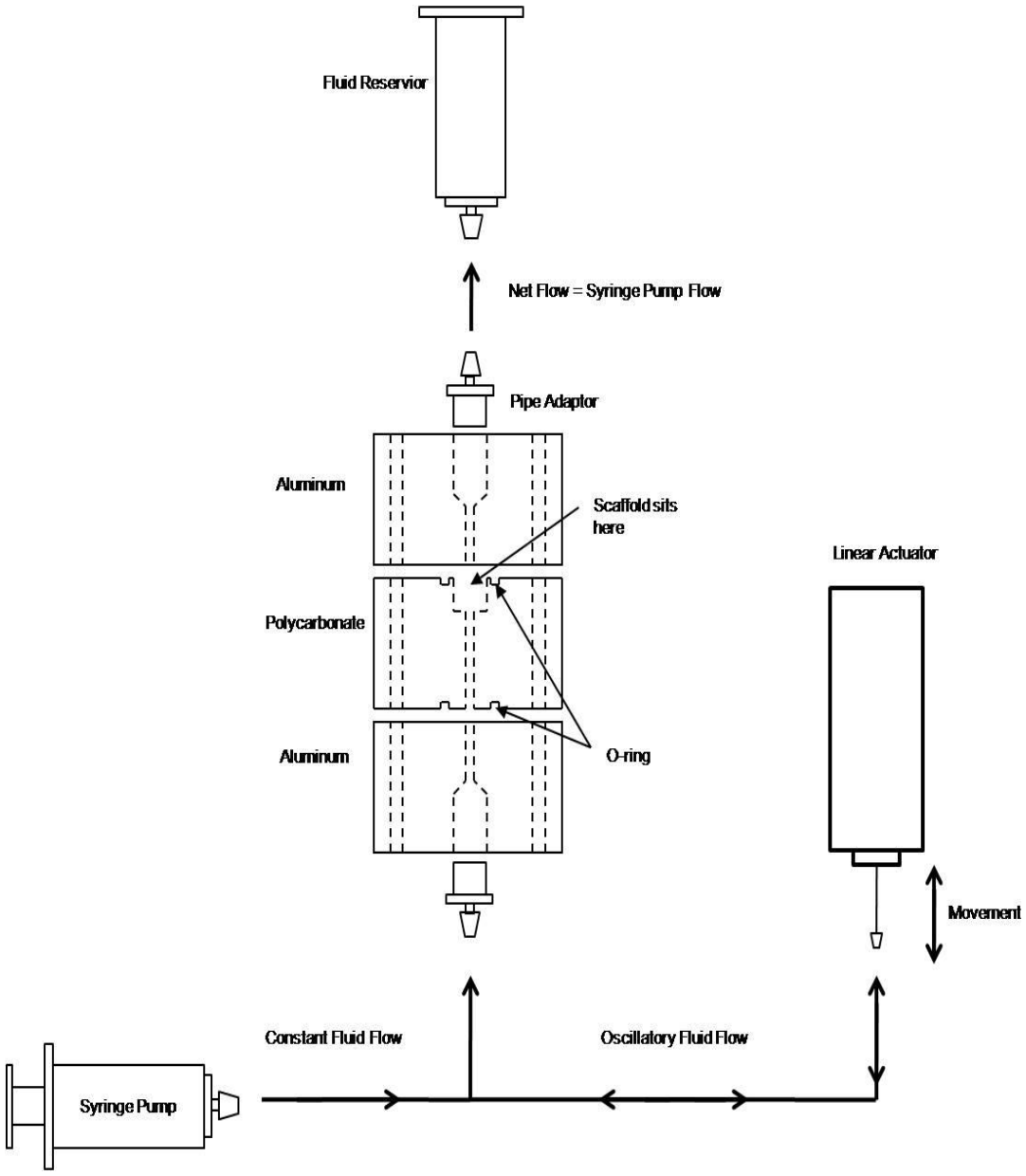

Figure 1 - Bioreactor setup for high-shear flow superimposed over low-shear flow. A syringe pump and a linear actuator were both in line with the bioreactor. Net fluid flow equaled the rate of the syringe pump. See text for more details.

was drilled out of the polycarbonate block to fit a porous calcium phosphate scaffold 5-mm in diameter and 3.5-mm in height (BD Biosciences, Bedford, MA). A 4-mm hole was drilled through the center of all three blocks to allow fluid flow through the scaffold. Grooves were machined around both the hole and the space on each side of the polycarbonate block to fit a #11 Viton O-ring (Allorings, Hampton Falls, NH). A $1/8^{th}$ inch barbed pipe connector was threaded into both the top and bottom aluminum blocks and connected to $1/8^{th}$ inch silicone tubing (Harvard Apparatus, Holliston, MA). The silicone tubing used is permeable to both CO_2 and O_2, so that cells were permitted adequate gas exchange. Tubing connected the top of the chamber to a 140 ml syringe fluid reservoir and the bottom of the chamber to a three-way valve. This valve was connected to both a syringe pump (Harvard Apparatus) and an electromagnetic linear actuator (Linmot, Delavan, WI). The syringe pump provided constant perfusion flow, while the linear actuator superimposed oscillatory flow at a higher shear rate. Once ready for experimentation, scaffolds were placed into the bioreactor chambers and connected into the flow circuit.

Experimental Design

My first 2 experiments were major experiments that were designed to establish the effects of partitioning mechanical stimulation on gene expression in osteoblasts seeded onto 3D scaffolds, and to compare those effects with single-bout mechanical stimulation as well as perfusion flow and static culture. The experiments were also designed to compare different incubation methods (static vs. perfusion). Following the first experiment, changes were made in the variable parameters (flow rates) to increase cellular response. The general design of the experiment did not change.

Experimental Design – Experiment 1

There were six experimental groups in addition to a static control and a vehicle control (eight total groups). The six experimental groups consisted of three loading treatments with two types of incubation after loading; a static incubation group and a perfusion incubation group. Each group contained 4 samples. Treatment groups can be seen in Table 1.

	60 min Perfusion Flow	60 min Oscillatory Flow Superimposed on Perfusion	4 - 15 min bouts Oscillatory Flow with 15 min Rest Periods between	Static Incubation	Perfusion Incubation
Static				x	
Vehicle				x	
Perfusion	x			x	
High-shear	x	x		x	
Partitioned High-shear	x		x	x	
Perfusion	x				x
High-shear	x	x			x
Partitioned High-shear	x		x		x

Table 1: Treatment groups included a static and vehicle control. There were three loading treatments: perfusion flow, high-shear oscillatory flow, and portioned high-shear oscillatory flow. Loading occurred for one hour followed by 24 hours of incubation. There were two incubation methods: static or perfusion.

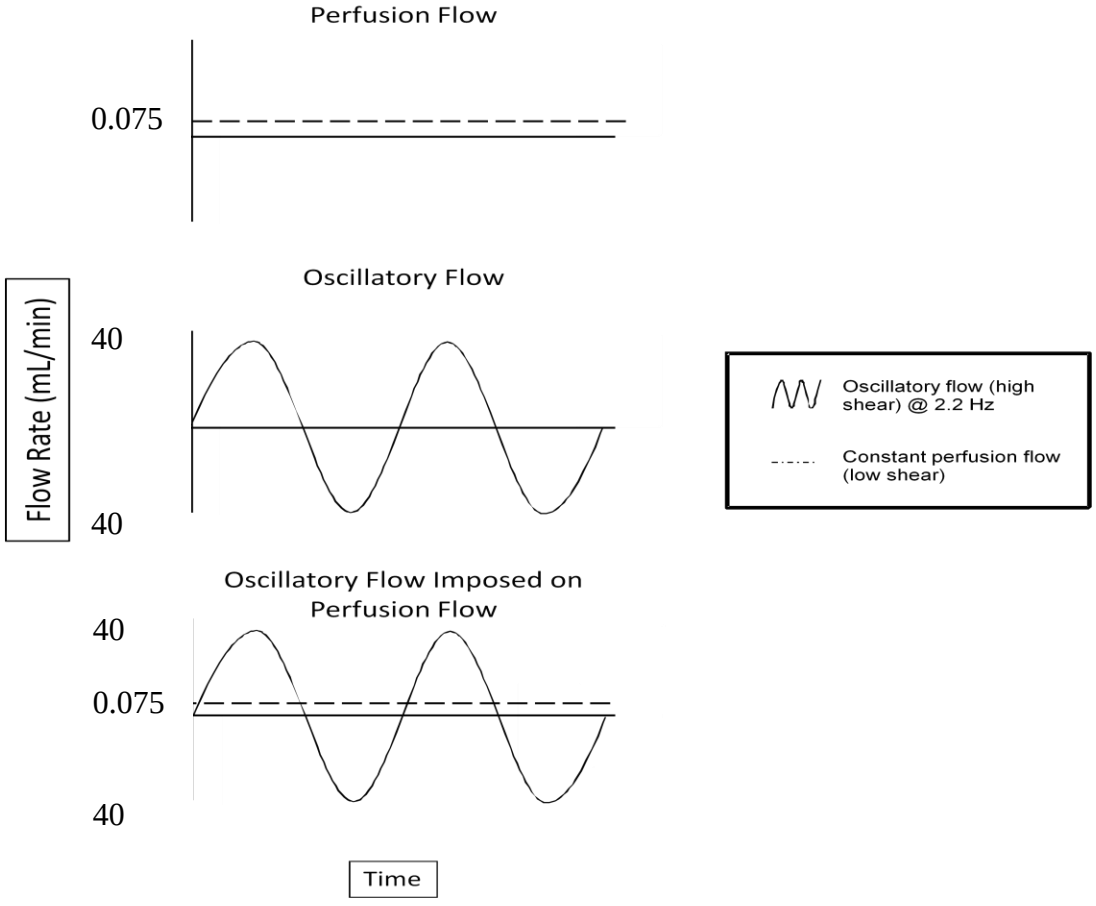

Figure 2 - Flow profiles for loading regimes. Perfusion flow remained at a constant flow rate, while oscillatory flow fluctuated sinusoidally. When oscillatory flow was superimposed over perfusion flow, the net flow was equal to the rate of perfusion flow.

The syringe pump delivered media at a rate of 0.025 mL/min (0.0006 Pa) for perfusion flow. This rate was not high enough to be within the range considered to be effective mechanical stimulation for bone cells (8), and was therefore considered to be low-shear. To deliver high-shear fluid flow (mechanical stimulation), the linear actuator was used to superimpose oscillatory flow on perfusion flow with a sinusoidal waveform at a maximum rate of 40 mL/min, with a frequency of 1 Hz. This rate created estimated shear stresses of ~1.0 Pa. Flow profiles for all loading regimes can be seen in Figure 2.

Oscillatory flow was applied immediately after perfusion was initiated and continued for either 1 hour (continuous loading) or was partitioned into four intermittent, fifteen minute bouts with fifteen minutes of rest between loadings. For static cultures, scaffolds remained in 24-well plates. All experimentation was performed in an incubator maintained at 37°C and 5% CO_2.

Total loading time for all mechanical treatments equaled one hour. After treatment (loading), scaffolds were removed from the bioreactor, placed into 24-well

Gene Name	**Function**	**Primer Sequence**
Osteopontin	Osteoblast Differentiation	F: 5'- CAGCTGGATGAACCAAGTCTGGAA -3'; R: 5'- ACTAGCTTGTCCTTGTGGCTGTGA -3'
Collagen Type I	Matrix Protein	F: 5'- TGGTTTGGAGAGAGCATGACCGAT -3'; R: 5'- TGTAGGCTACGCTGTTCTTGCAGT -3'
Vascular Endothelial Growth Factor A	Angiogenesis	F: 5'- ACAGAAGGAGAGCAGAAGTCCCAT -3'; R: 5'- ATGTGCTGGCTTTGGTGAGGTTTG -3'
COX-2	Inflammatory/ osteogenic	F: 5'- TCAATACTGGAAGCCGAGCACCTT -3'; R: 5'- GCACTTGCATTGATGGTGGCTGTT -3'
Cyclophillin	Housekeeping	F: 5'- TCATGTGCCAGGGTGGTGACTTTA; R: 5'- ATGCTTGCCATCCAGCCATTCAGT -3'
Beta-Actin	Housekeeping	F: 5'- ATCACTATTGGCAACGAGCGGTTC -3'; R: 5'- TCTCCTTCTGCATCCTGTCAGCAA -3'
Ubiquitin	Housekeeping	F:5'-CGTCGAGCCCAGTGTTACCACCAAGAAGG-3; R:5'-CCCCCATCACACCCAAGAACAAGCACAAG-3'

Table 2 – Primer design and function for genes of interest in partitioned mechanical study.

plates, covered with 2 mL of media, and statically incubated, or they remained in the bioreactor under constant perfusion flow for the remaining incubation time. At 25 hours after the onset of the loading treatment, RNA was isolated, and mRNA levels were determined for osteogenic and angiogenic genes (Table 2).

Experimental Design – Experiment 2

For the second experiment, conditions were changed to amplify cellular response. While the same treatment groups were used, flow rates were modified. The syringe pump delivered media at a new rate of 0.075 mL/min (~0.0017 Pa) for perfusion flow. The linear actuator superimposed oscillatory flow on perfusion flow with a sinusoidal waveform at a new maximum rate of 120 mL/min (2.2 Pa), at a frequency of 2.2 Hz. Twenty-five hours after the onset of loading (identical time point as experiment 1), mRNA levels for osteogenic and angiogenic genes were quantified.

RNA Isolation, RT-PCR

Scaffolds were placed in 2 ml tubes and crushed in SV Lysis Buffer with a glass rod. After lysis, dilution buffer was added along with 200 mM phosphate buffer to elute the RNA. RNA was isolated from cells using the Promega SV total RNA Isolation kit according to manufacturer's instructions. RNA concentration and quality was determined using spectrophotometer (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, Rockland, DE) readings at 260, 230, and 280 nm. Gel electrophoresis was used to verify RNA integrity. RNA was reverse transcribed into cDNA using a reaction mix consisting of Superscript II reverse transcriptase (Invitrogen, Calrsbad, CA), 1x first strand buffer (Invitrogen), 800 µM dNTPs (Promega), Rnase out recombinant ribonuclease inhibitor (Invitrogen), 48 mM dithiothreitol (Invitrogen) and 0.5 µg Oligo(dT)$_{12\text{-}18}$ primer at 42°C for 20 minutes, 50°C for 10 minutes and 42°C for 1 hour in the Mastercycler Gradient Thermocycler (Eppendorf, Westbury, NY). cDNA was then used for real time PCR for the genes of interest and the housekeeping gene (Table 1).

All reactions were performed in the StepOnePlus real-time PCR system (Applied Biosystems) under the following cycle parameters: hot start at 95°C for 15 minutes

followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds. Threshold fluorescence was set to 1500 dR and the C_t value was determined for all reactions. C_t values were used to determine the relative up or down regulation for each gene using the relative standard curve method and normalizing to one housekeeping gene that does not change between treatments (18).

Statistics

A two-factor (loading, incubation) analysis of variance (ANOVA) was used to test for model effects comparing mRNA levels. If significance was attained, follow-up post-hoc tests (students-T) were run to determine differences between pairs. JMP IN 5.1 statistical software package (SAS, Cary, NC) was used for all statistical analyses with a significance level of 0.05.

Results – Experiment 1

Loading had no effect on gene expression for any gene of interest. Even when one-factor ANOVAs were run within each incubation method (static vs. perfusion), neither perfusion flow nor mechanical stimulation (single bout or partitioned bouts) elicited changes in gene expression.

In contrast, incubation method affected gene expression (Figure 3) with perfusion

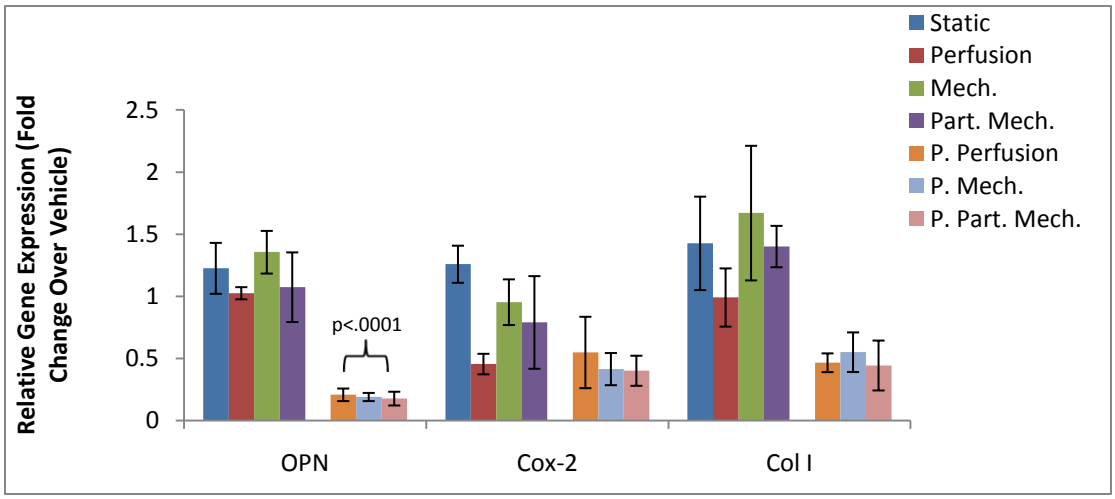

Figure 3 - Gene expression of OPN is down-regulated with perfusion incubation compared to static incubation. Loading method did not alter gene expression in any gene measured.

incubation down-regulating the gene expression of OPN by ~75% ($p < 0.0001$). No other genes had significant effects.

In this experiment, shear stresses experienced by cells attached to the scaffolds were estimated according to the following equations:

$$\tau_w = Q*x$$

where τ_w = average shear stress, Q = flow rate, and

$$x = 8*\mu/(d*(\Phi*\Pi(d/2)^2))$$

where μ is the viscosity of the culture medium (0.01 dyne-s/cm^2), d is the diameter of the average pore size, and Φ is the porosity.

These calculations suggested that at a high-shear oscillatory flow rate of 40 mL/min, cells would be subjected to shear stresses of ~1.0 Pa. This falls within the range of physiological shear stresses estimated to be experienced by osteoblasts in vivo. Low-shear perfusion flow at a rate of 0.025 mL/min causes shear stresses several orders of magnitude lower than in vivo estimations (0.0006 Pa).

Discussion

Mechanical stimulation had no effect on mRNA levels of any genes that were tested. As the equations estimating shear stress were developed for basic flow through a pipe, we decided to utilize a Computational Fluid Dynamics model to obtain a more accurate estimate of shear stresses experienced by cells within our scaffolds (19). Calculations suggested that at a flow rate of 40 mL/min, cells would only be subjected to shear stresses of ~0.7 Pa, rather than the 2.0 Pa originally calculated. Since shear stresses necessary to induce a response to mechanical stimulation in vivo have been estimated to be in the range of 0.8-3.0 Pa (20) we thought it necessary to increase the shear stress profile in future experiments. Therefore, a new rate of 120 mL/min was used, providing shear stresses of ~2.2 Pa (19).

Since literature has shown that perfusion flow increases many bone-related genes (OPN, Col1, etc) at later time points (e.g. 7 and 14 days) (3,4,21,22), we hypothesized that perfusion would increase those same genes at earlier time points (e.g. 24 hours) as well. Rather than up-regulating gene expression, perfusion incubation actually reduced OPN expression significantly. This was concerning as it suggested a potential reduction in osteoblast activity. We concluded that the rate of perfusion flow utilized in our bioreactor was not high enough to improve the oxygen and nutrient delivery necessary for increased osteoblast activity. Thus, we chose to increase the level of perfusion flow to better match values existing in literature.

Results – Experiment 2

Results for Experiment 2 were similar to Experiment 1. Loading had no effect on gene expression for any gene of interest ($p > 0.53$). Even when one-factor ANOVAs were run within each incubation method (static vs. perfusion), neither perfusion flow nor mechanical stimulation (single bout or partitioned bouts) elicited changes in gene expression.

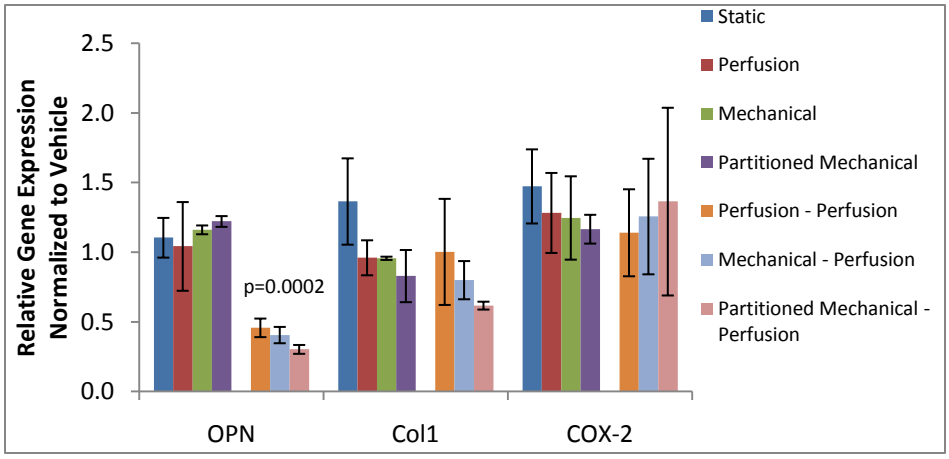

Figure 4 - OPN gene expression is down-regulated by perfusion incubation. Loading does not alter gene expression in static or perfusion incubation.

Here too, incubation method affected gene expression with perfusion incubation down-regulating the gene expression of OPN (Figure 4) by ~65% ($p = 0.0002$). No other genes had significant effects (Table 3).

	Model Effects	Loading	Incubation	Interaction
OPN *	0.0053	0.98	0.0002	0.59
VEGF	0.12	0.81	0.014	0.62
Col1	0.086	0.53	0.56	0.82
COX-2	0.99	0.99	0.95	0.89
Bcl-2/Bax	0.4	0.89	0.074	0.56

Table 3 - p-values for all genes with two-factor ANOVA. * = significant model effect. Only OPN was significant with perfusion flow decreasing OPN expression.

Discussion

As osteoblasts differentiate, they progress through a well-established pattern of gene expression, resulting in the deposition of calcium throughout a collagenous matrix (23). Alkaline phosphatase, osteopontin, and osteocalcin, each considered to be markers of osteoblast differentiation, follow a similar pattern of expression, increasing over the course of matrix maturation. ALP mRNA precedes OPN and OCN mRNA expression by approximately one week, peaking at approximately 21 days of culture in differentiation media. Collagen I expression peaks two week prior to ALP, and subsequently decreases in expression throughout the maturation process.

When using perfusion flow in 3D osteoblast cultures, literature demonstrates an increase in ALP, OPN, and OCN gene expression during long-term studies (7-14 days) (3,4,21,22). We expected to see early (24-48 hours) changes in gene expression that correlated to later time points presented in literature. As this did not occur, we concluded that either A) our bioreactor system did not provide adequate stimulation, or B) long-term gene expression may not predict similar patterns in early gene expression. A smaller, higher sample size experiment was designed to explore early patterns of gene expression in static incubation vs. perfusion incubation.

As mechanical stimulation is recognized as a major mediator of gene expression in osteoblasts, we hypothesized that high-shear oscillatory fluid flow (in single or multiple bouts) would increase gene expression in osteoblasts seeded in 3D. In 2D studies, mechanical stimulation has been shown to increase several of the genes we chose to measure (24-26), but there remains a paucity of data studying the effects of mechanical stimulation in 3D cultures, especially at early time points. Neither mechanical stimulation nor partitioned mechanical stimulation had any effects on gene expression during static incubation or perfusion incubation. To further elucidate the effects of mechanical stimulation in 3D, we designed another experiment that compared the effects of high-shear oscillatory flow on osteoblasts in both 2D and 3D.

In conclusion, under the conditions described, mechanical stimulation (single or multiple bouts) does not alter gene expression in 3D scaffolds after 24 hours of loading. However, 24 hours of perfusion incubation reduces the expression of OPN, suggesting a reduction in osteoblast activity.

References

1. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE 2003 Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng **9**(6)**:**1197-203.

2. Vance J, Galley S, Liu DF, Donahue SW 2005 Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release. Tissue Eng **11**(11-12)**:**1832-9.

3. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG 2002 Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A **99**(20)**:**12600-5.

4. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG 2003 Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci U S A **100**(25)**:**14683-8.

5. Holtorf HL, Jansen JA, Mikos AG 2005 Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone. J Biomed Mater Res A **72**(3)**:**326-34.

6. Rubin J, Rubin C, Jacobs CR 2006 Molecular pathways mediating mechanical signaling in bone. Gene **367:**1-16.

7. Iqbal J, Zaidi M 2005 Molecular regulation of mechanotransduction. Biochem Biophys Res Commun **328**(3)**:**751-5.

8. Weinbaum S, Cowin SC, Zeng Y 1994 A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech **27**(3)**:**339-60.

9. Robling AG, Burr DB, Turner CH 2000 Partitioning a daily mechanical stimulus into discrete loading bouts improves the osteogenic response to loading. J Bone Miner Res **15**(8)**:**1596-602.

10. Donahue SW, Donahue HJ, Jacobs CR 2003 Osteoblastic cells have refractory periods for fluid-flow-induced intracellular calcium oscillations for short bouts of flow and display multiple low-magnitude oscillations during long-term flow. J Biomech **36**(1)**:**35-43.

11. Batra NN, Li YJ, Yellowley CE, You L, Malone AM, Kim CH, Jacobs CR 2005 Effects of short-term recovery periods on fluid-induced signaling in osteoblastic cells. J Biomech **38**(9)**:**1909-17.

12. Holtorf HL, Datta N, Jansen JA, Mikos AG 2005 Scaffold mesh size affects the osteoblastic differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor. J Biomed Mater Res A **74**(2)**:**171-80.

13. Gomes ME, Holtorf HL, Reis RL, Mikos AG 2006 Influence of the porosity of starch-based fiber mesh scaffolds on the proliferation and osteogenic differentiation of bone marrow stromal cells cultured in a flow perfusion bioreactor. Tissue Eng **12**(4)**:**801-9.

14. Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG 2006 In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci U S A **103**(8)**:**2488-93.

15. Tanaka SM, Sun HB, Roeder RK, Burr DB, Turner CH, Yokota H 2005 Osteoblast responses one hour after load-induced fluid flow in a three-dimensional porous matrix. Calcif Tissue Int **76**(4)**:**261-71.

16. Cowin SC 2001 Bone mechanics handbook, 2nd ed. CRC Press, Boca Raton, FL, pp 1 v. (various pagings).

17. Cornell CN 1999 Osteoconductive materials and their role as substitutes for autogenous bone grafts. Orthop Clin North Am **30**(4)**:**591-8.

18. Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29**(9)**:**e45.

19. Jungreuthmayer C. JMJ, Donahue S.W.,, Al-Munajjed A.A. KDJ, O'Brien F.J. 2008 A comparison of collagen-GAG and calcium phosphate scaffolds subjected to fluid flow in a perfusion bioreactor- A 3D numerical fluid dynamics study. Abstract - The 14th Annual
Conference of the Section of Bioengineering.

20. Goldstein AS, Juarez TM, Helmke CD, Gustin MC, Mikos AG 2001 Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. Biomaterials **22**(11)**:**1279-88.

21. Jaasma MJ, O'Brien FJ 2008 Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A **14**(7)**:**1213-23.

22. Plunkett NA, Partap S, O'Brien FJ Osteoblast response to rest periods during bioreactor culture of collagen-glycosaminoglycan scaffolds. Tissue Eng Part A **16**(3)**:**943-51.

23. Stein GS, Lian JB 1993 Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev **14**(4)**:**424-42.

24. Barron M 2010 Mechanical Stimulation Mediates Gene Expression in MC3T3 Osteoblastic Cells Differently in 2D and 3D Environments. Journal of Biomechanical Engineering **132**.

25. You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR 2001 Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem **276**(16)**:**13365-71.

26. Jacobs CR, Yellowley CE, Davis BR, Zhou Z, Cimbala JM, Donahue HJ 1998 Differential effect of steady versus oscillating flow on bone cells. J Biomech **31**(11)**:**969-76.

CHAPTER THREE
2D vs. 3D

Mechanical Stimulation Mediates Gene Expression in MC3T3 Osteoblastic Cells Differently in 2D and 3D Environments

Matthew J. Barron, B.S., Chung-Jui Tsai, Ph.D., Seth W. Donahue, Ph.D.

Published in JOURNAL OF BIOMECHANICAL ENGINEERING, 2010

Journal of Biomechanical Engineering **132, 4**

Reprinted with Permission by the Publisher (See Appendix A)

Introduction

Bone tissue engineering is an expanding field that involves the use of three-dimensional (3D) constructs that could potentially be used as bone graft substitutes. While autografting is still the predominant choice for bone defect repair, the increasing popularity of synthetic bone grafts is driving the need for continued development in this area. To maximize the success of bone tissue engineering development, it is critical that we obtain a deeper understanding of cellular biology in an in vitro, three-dimensional architecture. Additionally, as perfusion bioreactors have become popular for 3D cell culture, it is necessary to understand the physical effects that fluid flow can have on cells in three dimensions.

Mechanical stimulation is recognized as a major mediator of both osteogenic and angiogenic activity in bone [1-6]. The adaptation of bone to mechanical loading is attributed to mechanotransduction, which is the conversion of mechanical stimuli into biochemical responses. In vivo, mechanical loading increases bone volume [7], bone strength [6, 8], bone formation rate [9], and angiogenic activity [10]. Prior to osteogenic endpoints, in vivo loading increases the gene expression of vital matrix proteins such as osteocalcin (OCN), collagen 1 (Col 1), and alkaline phosphatase (ALP) [11, 12]. Upstream of angiogenic activity, loading has been shown to augment the expression of vascular endothelial growth factor (VEGF) [13].

Historically, 2D in vitro cultures have played a key role in the study of bone mechanobiology. Subjecting bone cells to mechanical loading in 2D leads to increased expression of osteogenic genes such as OPN and Col 1 [14-19]. Similarly, loading in 2D also mediates angiogenic expression; osteoblast-like cells increase VEGF expression when exposed to either fluid flow [20] or mechanically-induced strain [21-23]. These 2D results (both osteogenic and angiogenic) parallel in vivo findings [11-13]. However, 2D experiments are performed on flat surfaces, which do not mimic in vivo 3D architecture. Thus, 3D in vitro models may provide more physiological environments for mechanotransduction studies.

Cells have been shown to react differently in 2D and 3D environments [24-27]. For example, both ALP and OCN (measures of osteoblast differentiation) were up-regulated in 3D cultures of human mesenchymal stem cells compared to those cultured in 2D [28, 29]. In another study, osteoblast-like cells exhibited enhanced angiogenic gene expression when cultured in 3D compared to those cultured in 2D [30]. Considering these results, it is clear that 2D models do not always accurately represent what will occur in 3D conditions.

Mechanical stimulation in 3D osteoblast cultures has not been studied extensively. However, there is literature showing that perfusion flow increases matrix production [31-33], mineralization [31-33], and late stage osteogenic gene expression [34, 35] in osteoblastic cells cultured on 3D scaffolds. Since the perfusion flow utilized in the these studies resulted in shear stresses well below those experienced in vivo [36], there remains little data on the effects of physiologically-relevant mechanical stimulation in 3D [3, 37]. Therefore, the goal of this study was to determine if flow-induced shear stress mediates osteogenic and angiogenic gene expression similarly in osteoblast-like cells cultured in 2D and 3D environments.

Materials and Methods

Cell Culture

Mouse osteoblast-like cells (MC3T3-E1 subclone 4, ATCC, Manassas, VA) between passage numbers 3 and 10 were cultured in alpha-MEM (Invitrogen, Carlsband,

CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Once cells reached ~70% confluency, they were trypsinized, re-suspended in culture medium, and statically seeded evenly onto either 2D glass slides (~ 7,000 cells/cm^2) or 3D calcium phosphate scaffolds (1,000,000 cells/scaffold) (BD Biosciences, San Jose, CA). The scaffolds have an interconnected porosity of ~ 60% with an average pore size of 200-400 microns. This is comparable to trabecular bone (50-90% porosity, 500-1500 micron pore size [38]), and within the range suggested to be optimal for bone regeneration (150 – 650 microns) in porous scaffolds [39]. Samples were then incubated for 1 hour to allow cell adhesion, and were covered with media and incubated for 24 hours before experiments began.

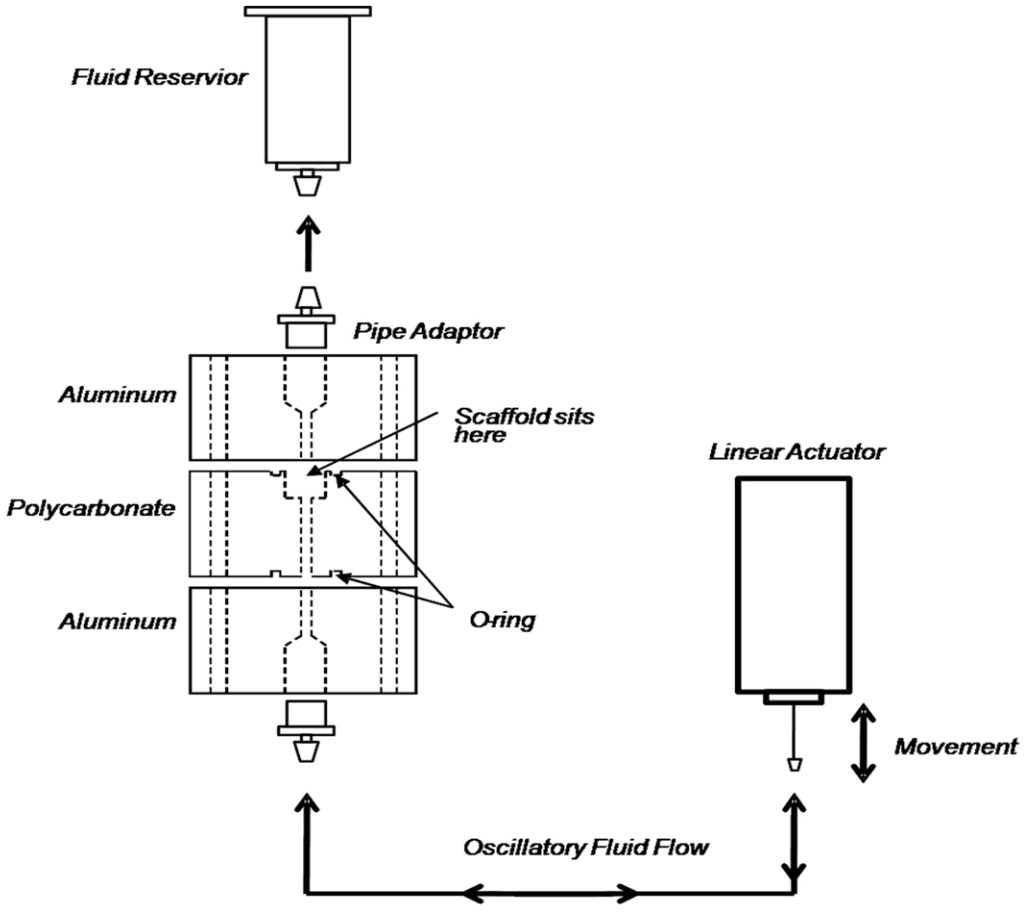

Figure 5 - Bioreactor setup with only the linear actuator in line. Only high-shear oscillatory fluid flow was used. See text for more details.

Mechanical Loading in 3D

A custom perfusion bioreactor was used to mechanically stimulate osteoblasts on calcium phosphate scaffolds. Each bioreactor chamber consists of a top and bottom aluminum block, between which a polycarbonate block is secured (Fig. 5). A space was drilled out of the polycarbonate block to fit a porous calcium phosphate scaffold 5-mm in diameter and 3.5-mm in height (BD Biosciences, Bedford, MA). A 4-mm hole was drilled through the center of all three blocks to allow fluid flow through the scaffold. Grooves were machined around both the hole and the space on each side of the polycarbonate block to fit a #11 Viton O-ring (Allorings, Hampton Falls, NH). A $1/8^{th}$ inch barbed pipe connector was threaded into both the top and bottom aluminum blocks and connected to $1/8^{th}$ inch silicone tubing (Harvard Apparatus, Holliston, MA). The silicone tubing used is permeable to both CO_2 and O_2, so that cells were permitted adequate gas exchange. Tubing connected the top of the chamber to a 140 ml syringe fluid reservoir. In addition, tubing connected the bottom of the chamber to an electromagnetic linear actuator (Linmot, Delavan, WI). The linear actuator imposed oscillatory flow at a controlled shear rate 2.2 Hz. Using these bioreactor and scaffold parameters, a computational fluid dynamics model was developed to estimate the shear stresses experienced by the cells within these scaffolds [40]. Calculations suggest that scaffolds subjected to a sinusoidal oscillatory flow profile at 120 ml/min will expose cells to an average shear stress of 2.2 Pa [40].

Mechanical Loading in 2D

For loading in 2D, cell-seeded glass slides were placed in a parallel-plate flow chamber (Streamer^{TM}, Flexcell International Corp, Hillsborough, NC) and connected to the same flow system. The linear actuator imposed oscillatory flow to expose cells to a shear stress of 2.2 Pa at a rate of 2.2 Hz.

Experimental Design

There were two model treatments (2D and 3D) and two loading treatments (static and mechanical). Each model/loading treatment combination contained 5 biological replicates. The static treatments remained incubated at 37°C with 5% CO_2 for the

duration of the experiment. The mechanical treatments were placed in their respective loading chambers and connected to the flow circuit. Loading occurred immediately after the circuit was connected and continued for 1 hour, after which they were removed and statically incubated for 23 hours. At this point, RNA was isolated, or samples were loaded a second time for 1 hour, followed by another 23 hour static incubation, after which RNA was isolated. Thus, 48 hour mechanical samples were loaded twice; for 1 hour at the beginning of each 24 hour period.

RNA Isolation, RT-PCR

For cells seeded on glass slides, the cells were trypsinized, pelleted, and lysed in SV Lysis Buffer. For cell-seeded scaffolds, samples were placed in 2 ml tubes and crushed in SV Lysis Buffer with a glass rod. After lysis, dilution buffer was added along with 200 mM phosphate buffer to elute the RNA. RNA was isolated from cells using the Promega SV total RNA Isolation kit according to manufacturer's instructions. RNA concentration and quality was determined using spectrophotometer (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, Rockland, DE) readings at 260, 230, and 280 nm. Gel electrophoresis was used to verify RNA integrity. RNA was reverse transcribed into cDNA using a reaction mix consisting of Superscript II reverse transcriptase (Invitrogen, Calrsbad, CA), 1x first strand buffer (Invitrogen), 800 µM dNTPs (Promega), Rnase out recombinant ribonuclease inhibitor (Invitrogen), 48 mM dithiothreitol (Invitrogen) and 0.5 µg Oligo(dT)12-18 primer at 42°C for 20 minutes, 50°C for 10 minutes and 42°C for 1 hour in the Mastercycler Gradient Thermocycler (Eppendorf, Westbury, NY). cDNA was then used for real time PCR for the genes of interest and the housekeeping gene (Table 4).

All reactions were performed in the StepOnePlus real-time PCR system (Applied Biosystems) under the following cycle parameters: hot start at 95°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds. Threshold fluorescence was set to 1500 dR and the Ct value was determined for all reactions. Ct values were used to determine the relative up or down regulation for each gene using the relative standard curve method and normalizing to one housekeeping gene that does not change between treatments [41].

Primer Name	Function	~Size (bp)	Primer Sequences
Collagen 1	Matrix protein	157	F: 5'- TGGTTTGGAGAGAGCATGACCGAT -3'; R: 5'- TGTAGGCTACGCTGTTCTTGCAGT -3'
Osteopontin	Matrix protein	158	F: 5'- CAGCTGGATGAACCAAGTCTGGAA -3'; R: 5'- ACTAGCTTGTCCTTGTGGCTGTGA -3'
COX-2	Osteogenic mediator	165	F: 5'- TCAATACTGGAAGCCGAGCACCTT -3'; R: 5'- GCACTTGCATTGATGGTGGCTGTT -3'
RUNX-2	Differentiation factor	200	F: 5'- AGAGTCAGATTACAGATCCCAGGC -3'; R: 5'- GTCAGAGGTGGCAGTGTCATCAT -3'
VEGF-A	Angiogenesis	261	F: 5'- ACAGAAGGAGAGCAGAAGTCCCAT -3'; R: 5'- ATGTGCTGGCTTTGGTGAGGTTTG -3'
FGF-2	Angiogenesis	150	F: 5'- AGCGGCTCTACTGCAAGAAC -3'; R: 5'- TGGCACACACTCCCTTGATA -3'
M-CSF 1	Angiogenesis	300	F: 5'- ATGGACACCTGAAGGTCCTG -3'; R: 5'- GCTGGAGAGGAGTCTCATGG -3'
Ubiquitin	Housekeeper	112	F: 5'-GTCGAGCCCAGTGTTACCACCAAGAAGG-3; R: 5'- CCCCCATCACACCCAAGAACAAGCACAAG-3'

Table 4: Primer sequences for 2D vs 3D study with high-shear oscillatory fluid flow.

Statistics

Five samples were tested for each of the 4 experimental conditions: 2D-Static, 2D-Mechanical, 3D-Static, 3D-Mechanical. A one way analysis of variance (ANOVA) was used to compare the mRNA levels between static cultures in 2D and 3D. A one way ANOVA was used to compare static and mechanical loading treatments within 2D and 3D cultures to determine the effects of mechanical stimulation in 2D and 3D. For graphical representation, gene expression was normalized to the average value of 2D Static samples. JMP IN 5.1 statistical software package (SAS, Cary, NC) was used for all statistical analyses with a significance level of 0.05.

Results

2D vs. 3D

There were several differences in gene expression between 2D and 3D under static culture, including OPN, Col1, FGF-2, and M-CSF (Table 5).

At 24 hours, OPN, a marker of osteoblast differentiation, and FGF-2, an angiogenic growth factor, were significantly ($p < 0.0009$) up-regulated in 3D by 22.5 and 4.1 fold, respectfully. In contrast, Col1, the most predominant bone matrix protein, and M-CSF, a chemotactic factor for macrophages (osteoclasts), were both down-regulated ($p < 0.0132$) in 3D, by 7.5 and 6.1 fold, respectively. RUNX2, COX-2, and VEGF were not significantly different ($p > 0.2839$).

Gene	Change in 3D	24 hour fold change	p-value at 24 hours	48 hour fold change	p-value at 48 hours
OPN	↑	22.5	0.0002	19.1	<0.0001
Col1	↓	7.5	0.0132	4.0	0.0211
FGF-2	↑	4.1	0.0009	10.3	0.0009
M-CSF	↓	6.1	0.0117	-	0.3034

Table 5: Fold changes in gene expression at 24 and 48 hours in static 3D osteoblast cultures relative to 2D cultures.

At 48 hours, there were also significant differences between 2D and 3D static treatments. Similar to 24 hours, OPN and FGF-2 were up-regulated in 3D at 19.1 and 10.3 fold ($p < 0.0009$). Col1 was down-regulated in 3D at 4.0 fold ($p = 0.0211$) (Table 5). There was no significant change in M-CSF, RUNX2, COX-2, or VEGF between 2D and 3D ($p > 0.3034$).

Static vs. Mechanical

No differences were seen with mechanical stimulation at 24 hours in either 2D ($p > 0.3514$) or 3D ($p > 0.5419$) when compared to static conditions. However, mechanical stimulation did have significant effects on gene expression at 48 hours, but the changes were not consistent between 2D and 3D models. OPN was up-regulated significantly ($p = 0.0302$) in 2D by ~ 50% (Fig. 6). In contrast, culture in 3D down-regulated OPN gene expression ($p = 0.0099$) by ~ 50% (Fig. 6).

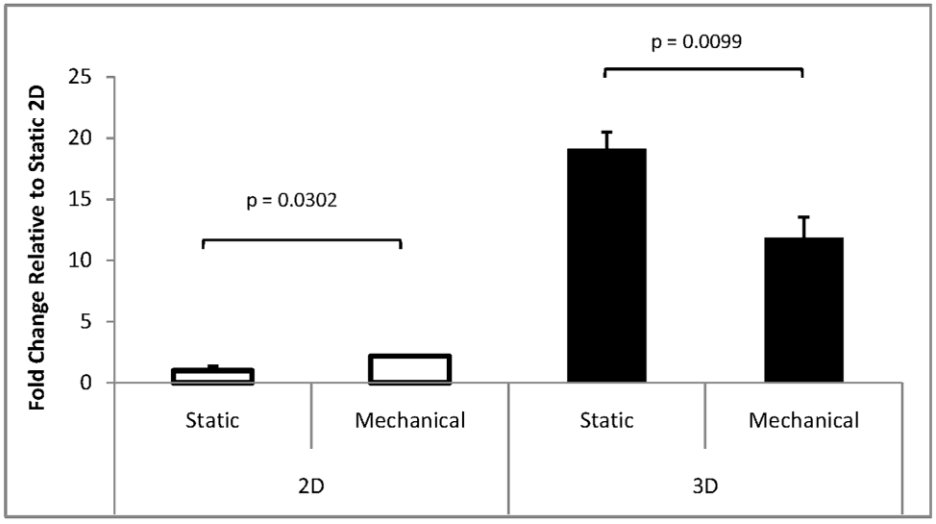

Figure 6: OPN gene expression at 48 hours in MC3T3 pre-osteoblasts after 1 hour of oscillatory fluid flow (per day) followed by 23 hours of static culture. Samples were loaded 2 times – once at the beginning of each 24 hour period. Represented as means (n=5) with standard error bars.

FGF-2, a potent angiogenic factor, was not affected ($p = 0.6165$) by mechanical loading in 2D cultures (Fig. 7), but was reduced ($p = 0.0691$) after mechanical stimulation in 3D. With COX-2, the limiting enzyme in the production of PGE2, mechanical loading in 2D had no effect ($p > 0.8519$). However, in 3D, COX-2 gene expression increased ($p = 0.0416$) when exposed to mechanical stimulation (Fig. 8). Col 1, RUNX-2, M-CSF, and VEGF-A were not affected by mechanical stimulation in 2D ($p > 0.3302$) or 3D ($p > 0.2847$) at 48 hours (data not shown).

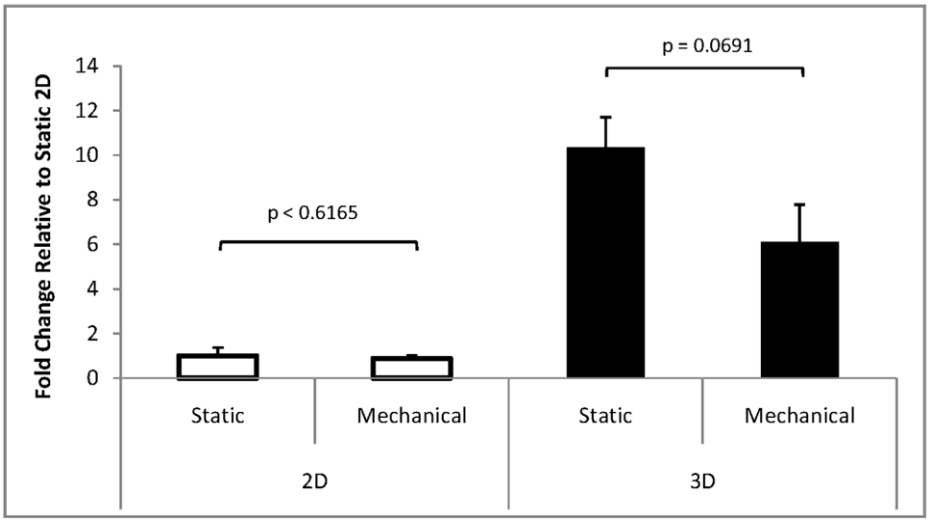

Figure 7: FGF-2 is down-regulated at 48 hours in 3D, but not 2D when osteoblasts are exposed to 1 hour of fluid flow (per day) followed by 23 hours of static culture. Represented as means (n = 5) with standard error bars.

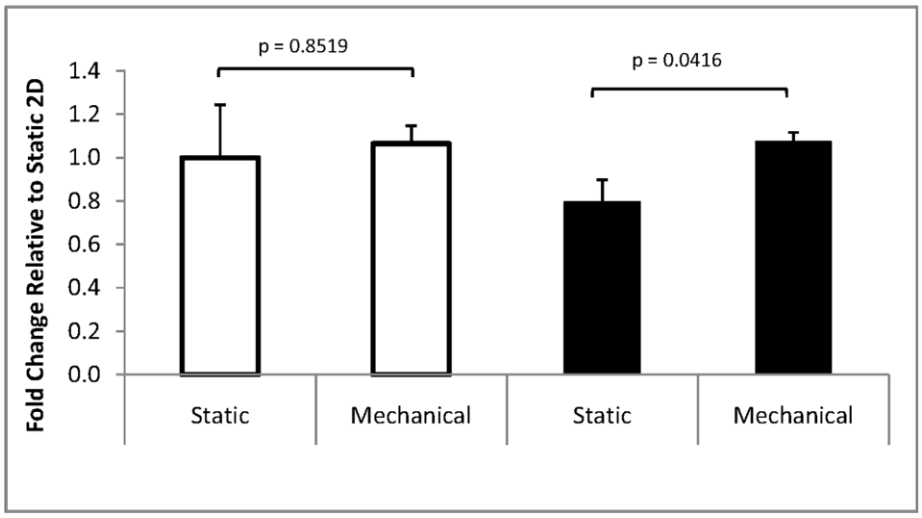

Figure 8: COX-2 is up-regulated significantly at 48 hours in 3D, but not 2D when osteoblasts are exposed to 1 hour of oscillatory fluid flow each day. Represented as means (n = 5) with standard error bars.

Discussion

The purpose of this study was to determine whether osteoblastic cells respond similarly to fluid-induced shear when cultured on 3D scaffolds and 2D glass slides. We found differential expression of several genes when comparing 3D and 2D cultures (Table 5). Moreover, when comparing effects of mechanical stimulation, 3D cultures responded differently than 2D cultures. These results support future work to identify the signaling pathways responsible for the different responses of osteoblast-like cells to mechanical stimulation on different architectures.

Our results showed different levels of gene expression between 2D and 3D osteoblast cultures under static conditions. At 24 and 48 hours, both OPN and FGF-2 were up-regulated in 3D, while collagen-1 was down-regulated in 3D (Table 5). One possibility for these differences in gene expression is that, under static incubation, 3D scaffolds experience a decrease in nutrient and oxygen delivery to the scaffold center. These conditions are consistent with those experienced under hypoxia, and are common in 3D constructs [42, 43]. It is know that OPN gene expression and FGF-2 protein production are increased under hypoxic conditions [44-47]. Interestingly, ERK1/2 is also activated under hypoxic conditions [48], and has been identified in the signaling pathways for both OPN and FGF-2. This important link suggests that when cultured

statically in 3D, osteoblasts may experience a hypoxia-driven increase in both OPN and FGF-2, possibly mediated by the ERK1/2 pathway. Furthermore, as Col 1 is decreased in MSC's under hypoxia [44], a decrease in Col 1 gene expression in the present study further suggests hypoxic conditions in static 3D cultures. Future experimentation measuring ERK1/2 or hypoxic activity (e.g. HIF-alpha expression) is necessary to determine whether hypoxia indeed plays a role in 3D osteoblast culture.

Previous studies suggest that OPN is up-regulated in pre-osteoblasts when exposed to mechanical stimulation; oscillatory flow up-regulated OPN by ~4 fold after 2 hours of stimulation, and by ~2.5 fold after 24 hours of stimulation in MC3T3 cells [15, 16]. In the present study, oscillatory flow up-regulated OPN in 2D cultures at both 24 and 48 hours (Fig. 6). However, significance was only achieved at 48 hours (2-fold, $p = 0.0302$). A larger sample size may establish significance at 24 hours, as OPN was up-regulated by 3-fold ($p = 0.3514$) when cells were exposed to mechanical stimulation. Contrasting 2D results, OPN was down-regulated ($p = 0.0099$) by oscillatory flow in 3D culture (Fig. 6) at 48 hours. Similarly, fluid flow also decreased FGF-2 ($p = 0.0691$), a potent angiogenic factor at 48 hours (Fig. 7). Like OPN, this result was unexpected as FGF-2 has been shown to be increased in osteoblasts after exposure to mechanical stimulation in 2D [23, 49, 50].

The different responses to mechanical loading in 2D and 3D cultures may be explained by hypoxic conditions induced by 3D culture. If OPN and FGF-2 are up-regulated in static 3D cultures because of a lack of oxygen delivery, fluid flow could counter this effect by delivering oxygen and down-regulating genes previously increased by the hypoxic state. If true, nutrient and oxygen delivery may supersede mechanotransduction in affecting OPN and FGF-2 gene expression in 3D. This suggests that different mechanisms drive cellular responses to fluid flow in 2D and 3D cultures.

One limitation of this study is the difference in substrates between 2D and 3D treatments. With glass as the substrate in our 2D model and calcium phosphate in our 3D model, it is possible that some of the changes in gene expression are due to differences in materials. In a 2D study comparing the effects of calcium phosphate-coated slides and plain glass slides, there were several changes in gene expression that were attributed to substrate. Bone sialoprotein, OPN, and RUNX2 were all up-regulated in human bone

marrow stromal cells when cultured on calcium phosphate (~2 fold, ~2 fold, ~1.5 fold respectively) compared to cells cultured on glass slides [51]. Thus, some of the changes in gene expression seen in our study may be due to substrate differences. However, we used the same calcium phosphate as the above study in our 3D scaffolds (Skelite™ - BD Biosciences). Since the fold changes seen in OPN in the present study (> 19 fold) were much greater than those seen in the 2D study above (~ 2 fold), we feel that the up-regulation in gene expression can be attributed to differences in architecture rather than substrate material. The increases in OPN levels of 23- and 19-fold at 24 and 48 hours (static comparison) in the present study are much greater than the 2-fold increase seen on calcium phosphate-coated slides in 2D [51]. Moreover, calcium phosphate did not elicit changes in Col 1 mRNA levels on 2D glass slides, whereas we saw significant decreases of 7- and 3.5- fold at 24 and 48 hours, respectively. Thus, the differences in gene expression in our study may be more strongly influenced by architecture than substrate.

Whether or not hypoxic conditions drive the distinct responses to substrate and fluid flow, it is clear that differences do exist between 2D and 3D cultures, suggesting that data collected in 2D studies may not be relevant to more complex 3D environments. Furthermore, when exposed to fluid flow in 3D, osteoblasts respond differently than when cultured in 2D. These results highlight the importance of future experimentation on mechanotransduction in 3D culture. Future research controlling the parameters of a 3D grafting structure, and studying in vitro cultures under dynamic conditions will provide critical information to define fundamental biochemical and biomechanical responses of bone cells.

The goal of bone tissue engineering is to produce 3D bone-like tissue in vitro that will respond functionally when implanted into the body. A major limitation is poor cellular viability in the center of 3D scaffolds. Our model may increase cellular viability in the center of 3D scaffolds through the reduction of hypoxic-like conditions. This would not only advance the quality of tissue-engineered bone, but should decrease culture times. Thus, utilizing fluid flow in a 3D bioreactor to stimulate osteoblast cultures holds promise for bone tissue engineering applications.

Acknowledgments

This publication was made possible through funding received from the National Science Foundation Graduate Research Fellowship Program.

References

[1] Rubin, J., Rubin, C., and Jacobs, C. R., 2006, "Molecular pathways mediating mechanical signaling in bone," Gene, 367, pp. 1-16.

[2] Iqbal, J., and Zaidi, M., 2005, "Molecular regulation of mechanotransduction," Biochem Biophys Res Commun, 328(3), pp. 751-755.

[3] Vance, J., Galley, S., Liu, D. F., and Donahue, S. W., 2005, "Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release," Tissue Eng, 11(11-12), pp. 1832-1839.

[4] Weiss, S., Zimmermann, G., Baumgart, R., Kasten, P., Bidlingmaier, M., and Henle, P., 2005, "Systemic regulation of angiogenesis and matrix degradation in bone regeneration--distraction osteogenesis compared to rigid fracture healing," Bone, 37(6), pp. 781-790.

[5] Li, G., Simpson, A. H., Kenwright, J., and Triffitt, J. T., 1999, "Effect of lengthening rate on angiogenesis during distraction osteogenesis," J Orthop Res, 17(3), pp. 362-367.

[6] Robling, A. G., Castillo, A. B., and Turner, C. H., 2006, "Biomechanical and molecular regulation of bone remodeling," Annu Rev Biomed Eng, 8, pp. 455-498.

[7] van der Meulen, M. C., Morgan, T. G., Yang, X., Baldini, T. H., Myers, E. R., Wright, T. M., and Bostrom, M. P., 2006, "Cancellous bone adaptation to in vivo loading in a rabbit model," Bone, 38(6), pp. 871-877.

[8] Robling, A. G., Burr, D. B., and Turner, C. H., 2000, "Partitioning a daily mechanical stimulus into discrete loading bouts improves the osteogenic response to loading," J Bone Miner Res, 15(8), pp. 1596-1602.

[9] Schriefer, J. L., Warden, S. J., Saxon, L. K., Robling, A. G., and Turner, C. H., 2005, "Cellular accommodation and the response of bone to mechanical loading," J Biomech, 38(9), pp. 1838-1845.

[10] Matsuzaki, H., Wohl, G. R., Novack, D. V., Lynch, J. A., and Silva, M. J., 2007, "Damaging fatigue loading stimulates increases in periosteal vascularity at sites of bone formation in the rat ulna," Calcif Tissue Int, 80(6), pp. 391-399.

[11] Moalli, M. R., Caldwell, N. J., Patil, P. V., and Goldstein, S. A., 2000, "An in vivo model for investigations of mechanical signal transduction in trabecular bone," J Bone Miner Res, 15(7), pp. 1346-1353.

[12] Pavlin, D., Zadro, R., and Gluhak-Heinrich, J., 2001, "Temporal pattern of stimulation of osteoblast-associated genes during mechanically-induced osteogenesis in vivo: early responses of osteocalcin and type I collagen," Connect Tissue Res, 42(2), pp. 135-148.

[13] Warren, S. M., Mehrara, B. J., Steinbrech, D. S., Paccione, M. F., Greenwald, J. A., Spector, J. A., and Longaker, M. T., 2001, "Rat mandibular distraction osteogenesis: part III. Gradual distraction versus acute lengthening," Plast Reconstr Surg, 107(2), pp. 441-453.

[14] Li, Y. J., Batra, N. N., You, L., Meier, S. C., Coe, I. A., Yellowley, C. E., and Jacobs, C. R., 2004, "Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation," J Orthop Res, 22(6), pp. 1283-1289.

[15] You, J., Reilly, G. C., Zhen, X., Yellowley, C. E., Chen, Q., Donahue, H. J., and Jacobs, C. R., 2001, "Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts," J Biol Chem, 276(16), pp. 13365-13371.

[16] Ponik, S. M., Triplett, J. W., and Pavalko, F. M., 2007, "Osteoblasts and osteocytes respond differently to oscillatory and unidirectional fluid flow profiles," J Cell Biochem, 100(3), pp. 794-807.

[17] Wu, C. C., Li, Y. S., Haga, J. H., Wang, N., Lian, I. Y., Su, F. C., Usami, S., and Chien, S., 2006, "Roles of MAP kinases in the regulation of bone matrix gene expressions in human osteoblasts by oscillatory fluid flow," J Cell Biochem, 98(3), pp. 632-641.

[18] Batra, N. N., Li, Y. J., Yellowley, C. E., You, L., Malone, A. M., Kim, C. H., and Jacobs, C. R., 2005, "Effects of short-term recovery periods on fluid-induced signaling in osteoblastic cells," J Biomech, 38(9), pp. 1909-1917.

[19] Sittichockechaiwut, A., Scutt, A. M., Ryan, A. J., Bonewald, L. F., and Reilly, G. C., 2009, "Use of rapidly mineralising osteoblasts and short periods of mechanical loading to accelerate matrix maturation in 3D scaffolds," Bone, 44(5), pp. 822-829.

[20] Thi, M. M., Iacobas, D. A., Iacobas, S., and Spray, D. C., 2007, "Fluid shear stress upregulates vascular endothelial growth factor gene expression in osteoblasts," Ann N Y Acad Sci, 1117, pp. 73-81.

[21] Singh, S. P., Chang, E. I., Gossain, A. K., Mehara, B. J., Galiano, R. D., Jensen, J., Longaker, M. T., Gurtner, G. C., and Saadeh, P. B., 2007, "Cyclic mechanical strain increases production of regulators of bone healing in cultured murine osteoblasts," J Am Coll Surg, 204(3), pp. 426-434.

[22] Motokawa, M., Kaku, M., Tohma, Y., Kawata, T., Fujita, T., Kohno, S., Tsutsui, K., Ohtani, J., Tenjo, K., Shigekawa, M., Kamada, H., and Tanne, K., 2005, "Effects of cyclic tensile forces on the expression of vascular endothelial growth factor (VEGF) and macrophage-colony-stimulating factor (M-CSF) in murine osteoblastic MC3T3-E1 cells," J Dent Res, 84(5), pp. 422-427.

[23] Fong, K. D., Nacamuli, R. P., Loboa, E. G., Henderson, J. H., Fang, T. D., Song, H. M., Cowan, C. M., Warren, S. M., Carter, D. R., and Longaker, M. T., 2003, "Equibiaxial tensile strain affects calvarial osteoblast biology," J Craniofac Surg, 14(3), pp. 348-355.

[24] Liu, H., Lin, J., and Roy, K., 2006, "Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells," Biomaterials, 27(36), pp. 5978-5989.

[25] Fischbach, C., Kong, H. J., Hsiong, S. X., Evangelista, M. B., Yuen, W., and Mooney, D. J., 2009, "Cancer cell angiogenic capability is regulated by 3D culture and integrin engagement," Proc Natl Acad Sci U S A, 106(2), pp. 399-404.

[26] Byrne, E. M., Farrell, E., McMahon, L. A., Haugh, M. G., O'Brien, F. J., Campbell, V. A., Prendergast, P. J., and O'Connell, B. C., 2008, "Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation," J Mater Sci Mater Med, 19(11), pp. 3455-3463.

[27] Farrell, E., Byrne, E. M., Fischer, J., O'Brien, F. J., O'Connell, B. C., Prendergast, P. J., and Campbell, V. A., 2007, "A comparison of the osteogenic potential of adult rat mesenchymal stem cells cultured in 2-D and on 3-D collagen glycosaminoglycan scaffolds," Technol Health Care, 15(1), pp. 19-31.

[28] Hishikawa, K., Miura, S., Marumo, T., Yoshioka, H., Mori, Y., Takato, T., and Fujita, T., 2004, "Gene expression profile of human mesenchymal stem cells during osteogenesis in three-dimensional thermoreversible gelation polymer," Biochem Biophys Res Commun, 317(4), pp. 1103-1107.

[29] Helmke, C., 1999, "Factors affecting bone cell growth and differentiation under differing culture conditions," Thesis, Rice University, Houston.

[30] Jarrahy, R., Huang, W., Rudkin, G. H., Lee, J. M., Ishida, K., Berry, M. D., Sukkarieh, M., Wu, B. M., Yamaguchi, D. T., and Miller, T. A., 2005, "Osteogenic differentiation is inhibited and angiogenic expression is enhanced in MC3T3-E1 cells cultured on three-dimensional scaffolds," Am J Physiol Cell Physiol, 289(2), pp. C408-414.

[31] Sikavitsas, V. I., Bancroft, G. N., Holtorf, H. L., Jansen, J. A., and Mikos, A. G., 2003, "Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces," Proc Natl Acad Sci U S A, 100(25), pp. 14683-14688.

[32] Bancroft, G. N., Sikavitsas, V. I., van den Dolder, J., Sheffield, T. L., Ambrose, C. G., Jansen, J. A., and Mikos, A. G., 2002, "Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner," Proc Natl Acad Sci U S A, 99(20), pp. 12600-12605.

[33] Holtorf, H. L., Jansen, J. A., and Mikos, A. G., 2005, "Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone," J Biomed Mater Res A, 72(3), pp. 326-334.

[34] Jaasma, M. J., and O'Brien, F. J., 2008, "Mechanical stimulation of osteoblasts using steady and dynamic fluid flow," Tissue Eng Part A, 14(7), pp. 1213-1223.

[35] Cartmell, S. H., Porter, B. D., Garcia, A. J., and Guldberg, R. E., 2003, "Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro," Tissue Eng, 9(6), pp. 1197-1203.

[36] Weinbaum, S., Cowin, S. C., and Zeng, Y., 1994, "A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses," J Biomech, 27(3), pp. 339-360.

[37] Tanaka, S. M., Sun, H. B., Roeder, R. K., Burr, D. B., Turner, C. H., and Yokota, H., 2005, "Osteoblast responses one hour after load-induced fluid flow in a three-dimensional porous matrix," Calcif Tissue Int, 76(4), pp. 261-271.

[38] Cowin, S. C., 2001, Bone mechanics handbook, CRC Press, Boca Raton, FL.

[39] Cornell, C. N., 1999, "Osteoconductive materials and their role as substitutes for autogenous bone grafts," Orthop Clin North Am, 30(4), pp. 591-598.

[40] Jungreuthmayer, C., Donahue, S. W., Jaasma, M. J., Al-Munajjed, A. A., Zanghellini, J., Kelly, D. J., and O'Brien, F. J., 2009, "A comparative study of shear stresses in collagen-glycosaminoglycan and calcium phosphate scaffolds in bone tissue-engineering bioreactors," Tissue Eng Part A, 15(5), pp. 1141-1149.

[41] Pfaffl, M. W., 2001, "A new mathematical model for relative quantification in real-time RT-PCR," Nucleic Acids Res, 29(9), p. e45.

[42] Mistry, A. S., and Mikos, A. G., 2005, "Tissue engineering strategies for bone regeneration," Adv Biochem Eng Biotechnol, 94, pp. 1-22.

[43] Griffith, C. K., Miller, C., Sainson, R. C., Calvert, J. W., Jeon, N. L., Hughes, C. C., and George, S. C., 2005, "Diffusion limits of an in vitro thick prevascularized tissue," Tissue Eng, 11(1-2), pp. 257-266.

[44] Potier, E., Ferreira, E., Andriamanalijaona, R., Pujol, J. P., Oudina, K., Logeart-Avramoglou, D., and Petite, H., 2007, "Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression," Bone, 40(4), pp. 1078-1087.

[45] Ishibashi, H., Nakagawa, K., Nakashima, Y., and Sueishi, K., 1995, "Conditioned media of carcinoma cells cultured in hypoxic microenvironment stimulate angiogenesis in vitro; relationship to basic fibroblast growth factor," Virchows Arch, 425(6), pp. 561-568.

[46] Gross, T. S., King, K. A., Rabaia, N. A., Pathare, P., and Srinivasan, S., 2005, "Upregulation of osteopontin by osteocytes deprived of mechanical loading or oxygen," J Bone Miner Res, 20(2), pp. 250-256.

[47] Raheja, L. F., Genetos, D. C., and Yellowley, C. E., 2008, "Hypoxic osteocytes recruit human MSCs through an OPN/CD44-mediated pathway," Biochem Biophys Res Commun, 366(4), pp. 1061-1066.

[48] Kroon, M. E., Koolwijk, P., van der Vecht, B., and van Hinsbergh, V. W., 2001, "Hypoxia in combination with FGF-2 induces tube formation by human microvascular endothelial cells in a fibrin matrix: involvement of at least two signal transduction pathways," J Cell Sci, 114(Pt 4), pp. 825-833.

[49] Li, C. F., and Hughes-Fulford, M., 2006, "Fibroblast growth factor-2 is an immediate-early gene induced by mechanical stress in osteogenic cells," J Bone Miner Res, 21(6), pp. 946-955.

[50] Cillo, J. E., Jr., Gassner, R., Koepsel, R. R., and Buckley, M. J., 2000, "Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: implications for distraction osteogenesis," Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 90(2), pp. 147-154.

[51] Scaglione, S., Wendt, D., Miggino, S., Papadimitropoulos, A., Fato, M., Quarto, R., and Martin, I., 2008, "Effects of fluid flow and calcium phosphate coating on human bone marrow stromal cells cultured in a defined 2D model system," J Biomed Mater Res A, 86(2), pp. 411-419.

CHAPTER FOUR
Perfusion Flow vs. Static Culture

Perfusion Flow Mediates Hypoxia-Specific Gene Expression in MC3T3 Osteoblastic Cells

Matthew J. Barron, B.S., Jeremy Goldman, Ph.D., Seth W. Donahue, Ph.D.

Introduction

Bone transplants are often necessary for spinal fusion, fracture healing, and defects resulting from trauma or disease (1-3). While autografts remain the best available option for bone grafting, the pain and morbidity associated with second surgeries combined with the limited availability of autograft sources drive the development of alternative grafting materials (1-3). Three-dimensional (3D) bone tissue engineering is promising to be an effective therapy for skeletal defect repair, with success defined by host acceptance, mechanical stability, and host tissue integration (1-3). 3D bone tissue engineering combines porous synthetic scaffolds, which allow tissue in-growth (osteoconductive), with growth factors to induce bone growth (osteoinductive) and/or osteoblasts (or mesenchymal stem cells) to produce bone (osteogenic). While this approach has seen success, a common limitation is the maintenance of cellular viability within the center of 3D constructs (6,7,13).

The delivery of oxygen and nutrients and the removal of waste are essential for cellular survival. Because of oxygen's poor diffusion capacity, oxygen transport largely governs the production of 3D tissue cultures *in vitro* (4,5). Perfusion bioreactors mitigate hypoxic (oxygen-depleted) conditions, as they provide a means of oxygen delivery to the center of 3D cell-seeded scaffolds (4). By constantly perfusing culture media through porous constructs, bioreactor systems have increased cellular infiltration and viability within 3D scaffolds by increasing the level of oxygen transport (4,6-10). This leads to up-regulation in gene expression (6,8,11), increases in protein production (7,10,12-15), and enhanced calcium deposition (7,13,16-18) in osteoblast-seeded scaffolds.

It is clear that hypoxia must be given consideration in tissue engineering applications. Given that perfusion bioreactors increase oxygen transport to 3D scaffolds (4), and that hypoxia restricts osteoblast differentiaton (19-22), it is reasonable to hypothesize that some of the benefit associated with the use of perfusion flow (6-8,10-18) may be attributed to the reduction of hypoxia in 3D culture. As most studies have focused on the ability of 3D perfusion systems to produce higher quality bone, few studies have sought to elucidate the molecular mechanisms behind the success of perfusion bioreactors. Thus, the goal of this study was to determine if hypoxia-specific gene expression in osteoblast-like cells cultured in a 3D scaffolds is affected by perfusion flow. We hypothesized that perfusion flow would reduce hypoxia-specific gene expression and increase cellular infiltration in osteoblast-seeded 3D scaffolds.

Materials and Methods

Cell Culture

Mouse osteoblast-like cells (MC3T3-E1 subclone 4, ATCC, Manassas, VA) between passage numbers 3 and 10 were cultured in alpha-MEM (Invitrogen, Carlsband, CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Once cells reached ~70% confluency, they were trypsinized, re-suspended in culture medium, and statically seeded on 3D calcium phosphate scaffolds (1,000,000 cells/scaffold) (BD Biosciences, San Jose, CA). The scaffolds have an interconnected porosity of ~ 60% with an average pore size of 200-400 microns. This is comparable to trabecular bone (50-90% porosity, 500-1500 micron pore size (23)), and within the range suggested to be optimal for bone regeneration (150 – 650 microns) in porous scaffolds (24). Samples were then incubated for 1 hour to allow cell adhesion, and were covered with media and incubated for 24 hours before experiments began.

Perfusion Flow Bioreactor

A custom perfusion bioreactor was used to deliver culture media to osteoblasts on calcium phosphate scaffolds. Each bioreactor chamber consists of a top and bottom aluminum block, between which a polycarbonate block is secured (Figure 9). A 6-mm

hole was drilled out of the polycarbonate block to fit a porous calcium phosphate scaffold 5-mm in diameter and 3.5-mm in height (BD Biosciences, Bedford, MA). A 4-mm hole was drilled through the center of all three blocks to allow fluid flow through the scaffold. Grooves were machined around both the hole and the space on each side of the polycarbonate block to fit a #11 Viton O-ring (Allorings, Hampton Falls, NH). A $1/8^{th}$ inch barbed pipe connector was threaded into both the top and bottom aluminum blocks and connected to $1/8^{th}$ inch silicone tubing (Harvard Apparatus, Holliston, MA). The silicone tubing is permeable to CO_2 and O_2, permitting adequate gas exchange. Tubing connected the top of the chamber to a 140 ml syringe fluid reservoir, and the bottom of the chamber to syringe pump (Harvard Apparatus). The pump delivered media at a rate of 0.075 ml/min (0.00017 Pa), with media being replaced each day.

Experimental Design – Gene Expression

Cell seeded scaffolds were cultured statically in an incubator or with perfusion flow in the bioreactor. The static samples remained incubated at 37°C with 5% CO_2 for the duration of the experiment. The perfusion samples were cultured in the bioreactor chambers. Perfusion flow was initiated immediately after the samples were placed in the chamber and continued for the remainder of the experiment. Samples were exposed to flow for 24 or 48 hours (n=5 per time point). Static samples were also incubated for 24 or 48 hours (n=5 per time point). After 24 or 48 hours of culture, the samples were removed from static or perfusion incubation and RNA was isolated.

RNA Isolation and RT-PCR

After culture, cell-seeded scaffolds were placed in 2 ml tubes and crushed in SV Lysis Buffer with a glass rod. After lysis, dilution buffer was added along with 200 mM phosphate buffer to elute the RNA. RNA was isolated from cells using the Promega SV total RNA Isolation kit according to manufacturer's instructions. RNA concentration and quality was determined using spectrophotometer (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, Rockland, DE) readings at 260, 230, and 280 nm. Gel electrophoresis was used to verify RNA integrity. RNA was reverse transcribed into cDNA using a reaction mix consisting of Superscript II reverse transcriptase (Invitrogen,

Calrsbad, CA), 1x first strand buffer (Invitrogen), 800 µM dNTPs (Promega), Rnase out recombinant ribonuclease inhibitor (Invitrogen), 48 mM dithiothreitol (Invitrogen) and 0.5 µg Oligo(dT)$_{12-18}$ primer at 42°C for 20 minutes, 50°C for 10 minutes and 42°C for 1 hour in the Mastercycler Gradient Thermocycler (Eppendorf, Westbury, NY). cDNA was then used for real time PCR for the genes of interest and the housekeeping gene (Table 6).

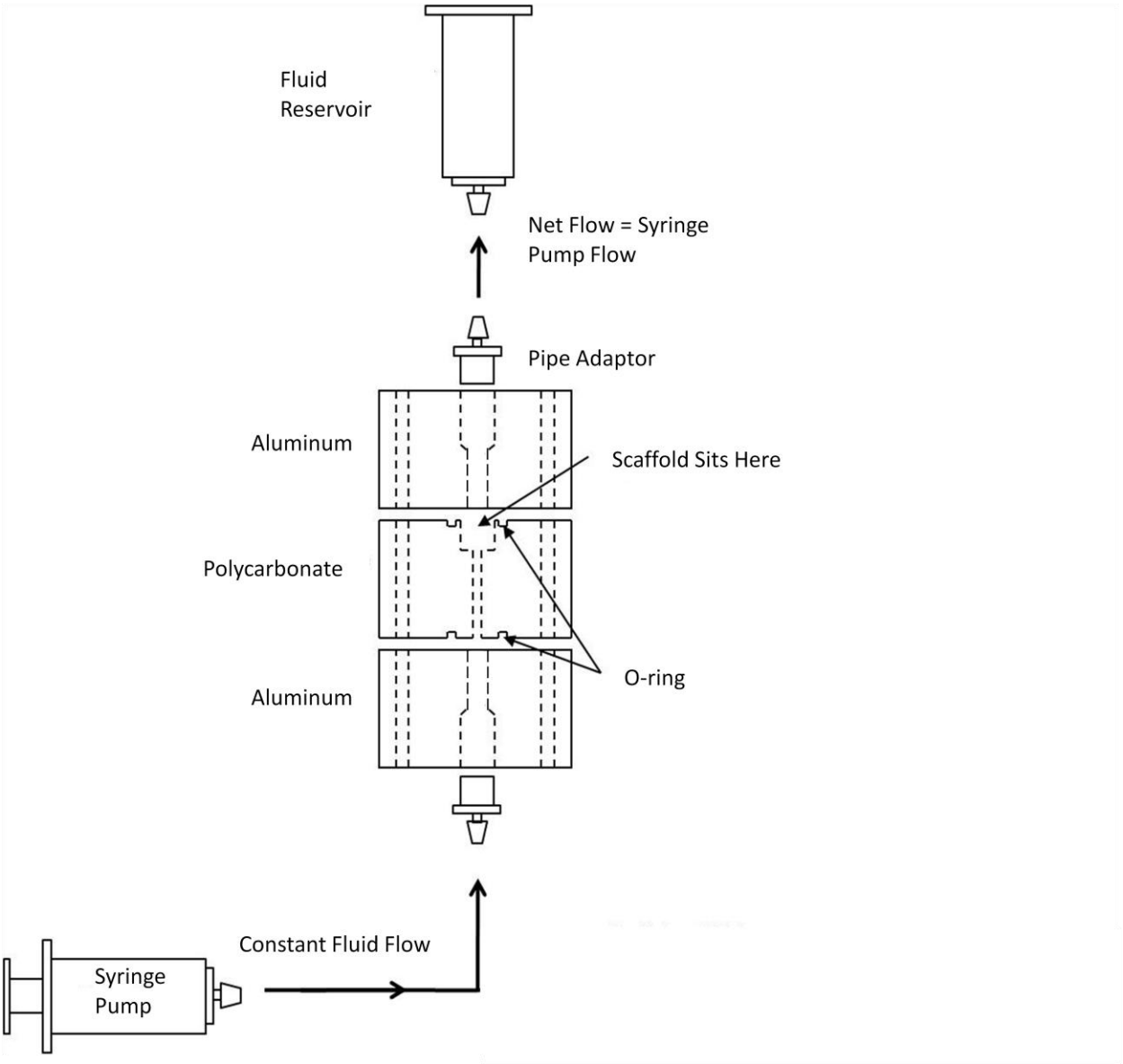

Figure 9 - Bioreactor setup for perfusion incubation. The linear actuator was not used in this study. The syringe pump delivered fluid at a rate of 0.075 mL/min. See text for more detail.

Gene Name	Function	Primer Sequence
Osteocalcin	Osteoblast Differentiation	F: 5'- GAGTCTGACAAAGCCTTCATGTCC -3'; R: 5'- TGATAGCTCGTCACAAGCAGGGTTA -3'
Osteopontin	Osteoblast Differentiation	F: 5'- CAGCTGGATGAACCAAGTCTGGAA -3'; R: 5'- ACTAGCTTGTCCTTGTGGCTGTGA -3'
Alkaline Phosphatase	Osteoblast Differentiation	F: 5'-GCCCTCTCCAAGACATATA-3'; R: 5'-CCATGATCACGTCGATATCC-3'
RUNX-2	Osteoblast Differentiation	F: 5'- AGAGTCAGATTACAGATCCCAGGC -3'; R: 5'- GTCAGAGGTGGCAGTGTCATCAT -3'
Collagen Type I	Matrix Protein	F: 5'- TGGTTTGGAGAGAGCATGACCGAT -3'; R: 5'- TGTAGGCTACGCTGTTCTTGCAGT -3'
Vascular Endothelial Growth Factor A	Angiogenesis	F: 5'- ACAGAAGGAGAGCAGAAGTCCCAT -3'; R: 5'- ATGTGCTGGCTTTGGTGAGGTTTG -3'
Basic Fibroblast Growth Factor 2	Angiogenesis	F: 5'- AGCGGCTCTACTGCAAGAAC -3'; R: 5'- TGGCACACACTCCCTTGATA -3'
Macrophage Colony Stimulating Factor	Angiogenesis	F: 5'- ATGGACACCTGAAGGTCCTG -3'; R: 5'- GCTGGAGAGGAGTCTCATGG -3'
COX-2	Inflammatory/ osteogenic	F: 5'- TCAATACTGGAAGCCGAGCACCTT -3'; R: 5'- GCACTTGCATTGATGGTGGCTGTT -3'
HIF-1α	Hypoxia	F: 5'- AAACTTCTGGATGCCGGTGGTCTA -3' R: 5'- TCT CAC TGG GCC ATT TCT GTG TGT -3'
GBE-1	Hypoxia	F: 5'- GCAGGTATAAGAAGTTTAGCCAGG -3' R: 5'- GAGAAAATGGATTCCAACCACTGAA -3'
MIF	Hypoxia	F: 5'- CGCACAGTACATCGCAGTG-3' R: 5'- CAG CGG TGC AGG TAA GTG-3'
Cyclophillin	Housekeeping	F: 5'- TCATGTGCCAGGGTGGTGACTTTA; R: 5'- ATGCTTGCCATCCAGCCATTCAGT -3'
Beta-Actin	Housekeeping	F: 5'- ATCACTATTGGCAACGAGCGGTTC -3'; R: 5'- TCTCCTTCTGCATCCTGTCAGCAA -3'
Ubiquitin	Housekeeping	F: 5'- CGTCGAGCCCAGTGTTACCACCAAGAAGG -3; R: 5'- CCCCCATCACACCCAAGAACAAGCACAAG -3'

Table 6 - Primer sequences and roles for genes measure in static vs. perfusion study.

All reactions were performed in the StepOnePlus real-time PCR system (Applied Biosystems) under the following cycle parameters: hot start at 95°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds. Threshold fluorescence was set to 1500 dR and the C_t value was determined for all reactions. C_t values were used to determine the relative up or down regulation for

each gene using the relative standard curve method and normalizing to one housekeeping gene that does not change between treatments (25).

Experimental Design – Long-term Cellular Survival

The cell-seeded scaffolds that received static treatment remained incubated at 37°C with 5% CO_2 for the duration of the experiment. Cell-seeded scaffolds that received perfusion treatments were placed into bioreactor chambers. Perfusion flow was initiated immediately after the circuit was connected and continued for 14 days. Following 14 days of culture, samples were removed from static or perfusion incubation and were fixed in 10% buffered formalin for histological processing.

Cell Number, Surface Area Coverage, and Cell/Matrix Area Coverage

Following fixation, 14-day samples were placed in cassettes, covered in OCT freeze medium, and placed under vacuum for 48 hours to remove air bubbles. Samples were then removed, flash frozen, and sectioned on a soft tissue microtome (Microm International, Waldorf

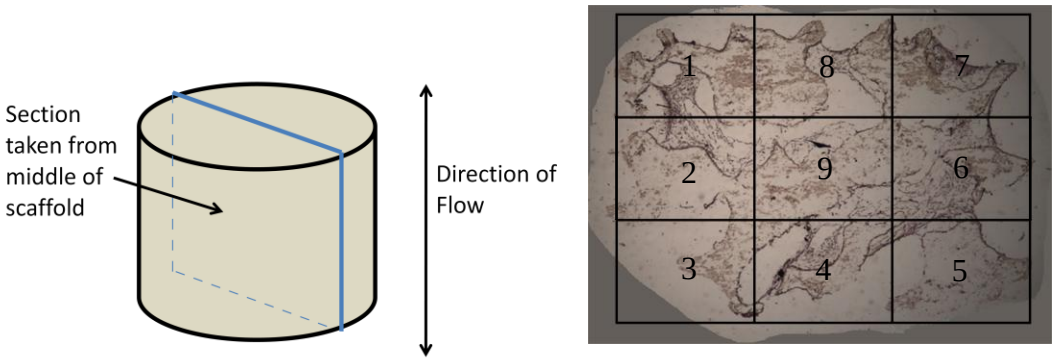

Figure 10 - Sections were removed from the middle of the scaffold parallel to the direction of flow. Nine sample regions from each section were used to quantify cell number, surface area coverage, and cell/matrix area coverage. Regions 1-8 are combined and are referred to as the scaffold perimeter. Region 9 is referred to as the scaffold center.

Germany) at a thickness of 10 microns. Sections were taken from mid-way through the scaffold running parallel to the direction of perfusion flow (Figure 10). Samples were then decalcified in 0.5% EDTA, stained with hematoxylin and eosin, and images were captured for the entire section from each sample at 100x total magnification. Nine

images were taken in total, three from the upper, middle, and lower thirds of each section (Figure 10). For cell number, surface area coverage and cell/matrix area coverage, measurements were taken from the scaffold perimeter (regions 1-8 combined) and the scaffold center (region 9).

Average cell number was determined by counting the total cell number in each region within a section. For the scaffold perimeter, cell number was calculated from each of the 8 regions and averaged so comparison could be made against the scaffold center (region 9 – Figure 10).

Surface area coverage (represented by % total perimeter) was calculated by measuring the ratio of cell coverage around the surface area of scaffold pores to the total surface area of scaffold pores (including outer surface) using Bioquant (Bioquant Osteo, Nashville, TN) software. Since surface area coverage was calculated as a percentage of total coverage area, so averaging all 8 regions was not necessary to compare with the scaffold center (region 9 – Figure 10).

Cell/matrix area coverage (represented as % pore space) was established by measuring the ratio of cell and matrix area within pore spaces to total pore space using a standard threshold value in Bioquant (Figure 11). Since cell/matrix area coverage was calculated as a percentage of total coverage area, so averaging all 8 regions was not necessary to compare with the scaffold center (region 9 – Figure 10).

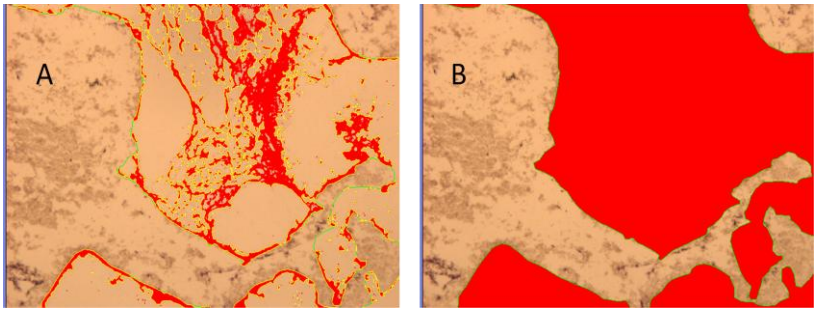

Figure 11 - To determine cellular infiltration, a ratio was calculated between A) area of cell/matrix coverage (thresholded red) and B) total area of pore spaces (thresholded red). The area in red was used for measurement and was determined by setting a threshold value in Bioquant.

Statistics

Three (cell number and infiltration) or five (gene expression) samples were tested for each of the 2 experimental conditions: Static and Perfusion. For cell number, surface area coverage and cell/matrix area coverage, measurements were taken from the scaffold perimeter (regions 1-8 combined) and the scaffold center (region 9). A one way analysis of variance (ANOVA) was used to compare mRNA levels (gene exression), average cell number, surface area coverage, and cell/matrix area coverage between static and perfusion cultures for both the scaffold perimeter (regions 1-8) and the scaffold center (region 9). JMP IN 5.1 statistical software package (SAS, Cary, NC) was used for all statistical analyses with a significance level of 0.05.

Results

Gene Expression

After 24 hours of flow, OPN (a marker of both bone development and the inflammatory response) was down-regulated ($p = 0.03$) by 10%, as was VEGF (a major angiogenic growth factor) by 48% ($p = 0.02$). In contrast, COX-2, another marker of both bone development and the inflammatory response, was up-regulated ($p = 0.02$) by perfusion flow by 67% (Table 7).

After 48 hours of perfusion flow, while the same trend existed for OPN, VEGF, and COX-2 as compared to 24 hours, the responses were not statistically significant ($p>0.20$) However, HIF-1α, the major transcription factor driving the hypoxic response, was down-regulated (0.02) by 55% (Table 7).

Gene	**24 hour change**	**p-value**	**48 hour change**	**p-value**
OPN	↓10%	0.03	-	-
COX-2	↑67%	0.02	-	-
VEGF	↓48%	0.02	-	-
HIF-1 α	-	-	↓45%	0.02

Table 7 - Changes in osteoblast gene expression when exposed to perfusion flow - hypoxia-related genes are down-regulated.

Runx-2, Col1, M-CSF, bFGF, and GBE-1 were not significantly different between Perfusion and Static treatments at either time point ($p > 0.20$).

Cell Number, Surface Area Coverage, and Cell/Matrix Area Coverage

Perfusion flow increased cell number by 1.5-fold ($p = 0.04$) around the scaffold perimeter (Figure 12). Perfusion also increased cell number in the scaffold center, although by a bigger margin of nearly 5-fold ($p = 0.04$), suggesting that perfusion flow has an amplified effect in areas of poor static diffusion (Figure 12).

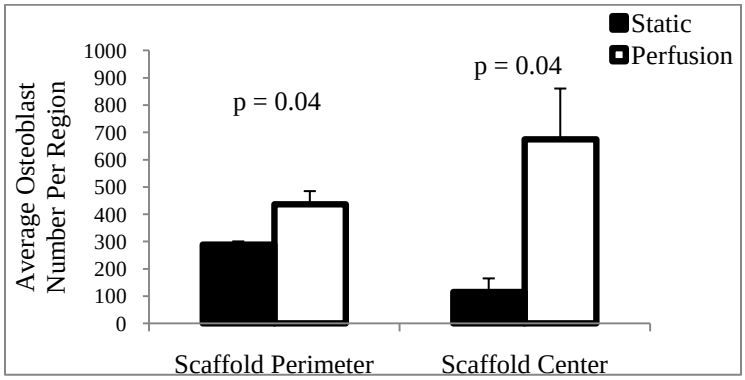

Figure 12 - Perfusion flow increases cell number after 14 days of culture.

Surface area coverage was not affected by perfusion flow around the scaffold perimeter ($p = 0.25$) or in the scaffold center ($p = 0.56$).

Cell/matrix area coverage was only increased by perfusion flow in the scaffold center (10-fold), further supporting the hypothesis that the scaffolds' centers are more sensitive to perfusion flow that the outer perimeter (Figure 13). These results are consistent with both literature and our hypothesis.

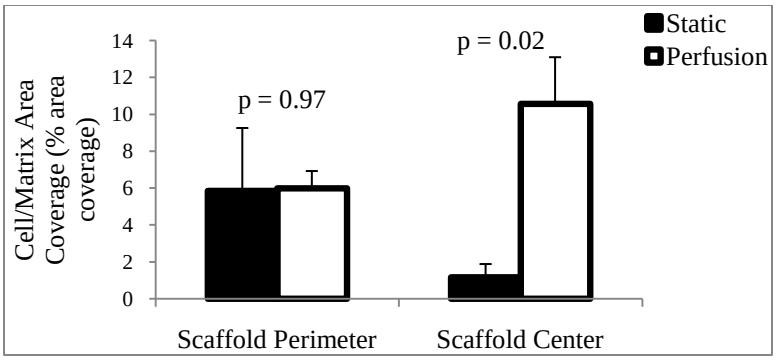

Figure 13 – Perfusion flow increases cell/matrix area coverage in the center of 3D scaffolds after 14 days of culture

Discussion

The goal of bone tissue engineering is to produce a 3D construct with high cellular viability and quality tissue structure. It is clear that hypoxia plays a role in limiting successful *in vitro* culture of large 3D structures. Previous research has shown that perfusion bioreactors produce higher-quality bone tissue *in vitro*, and reduce hypoxia by delivering oxygen to the center of 3D scaffolds (4,7,13). We found that cell number and cellular infiltration are both increased through the use of a perfusion bioreactor. We also found that osteoblasts down-regulated gene expression of VEGF, HIF-1α, and OPN when exposed to perfusion flow, suggesting perfusion flow rescues osteoblasts within 3D structures from hypoxic-like conditions.

Hypoxia elicits a well-documented response from mesenchymal stem cells and osteoblasts. Upon exposure to reduced oxygen tension, MSC's and osteoblasts up-regulate the exression of vascular endothelial growth factor (VEGF) and basic-fibroblast growth factor (bFGF), both important angiogenic genes (19,26). OPN, which is involved in the inflammatory response as well as bone development, is also up-regulated under hypoxic conditions (19,27). Since we have shown that perfusion flow decreases several of the genes that become activated during hypoxia, it is reasonable to hypothesize that perfusion bioreactors mitigate hypoxic conditions by providing adequate oxygen delivery.

OPN is commonly used as a benchmark endpoint in long-term 3D osteoblast studies. Most 3D bioreactor studies (7,8,13) demonstrate that OPN is up-regulated with

perfusion flow. In contrast, we showed that OPN is down-regulated with perfusion flow (24-hour time point). Because the expression of several genes were diminished in the perfusion treatment, we initially thought that total cellular activity may have been reduced under perfusion. However, subsequent long-term experiments refuted this, as relative cell number and cellular infiltration were both increased in perfusion flow samples. Additionally, ongoing work in our lab shows that bone specific gene expression (ALP, OCN) is indeed up-regulated at later time points (7 days) in 3D scaffolds exposed to perfusion flow, while VEGF (major regulator of hypoxia) remains down-regulated (OPN gene expression was not significantly different). This suggests that OPN may play a dual role in 3D tissue engineering. Indeed, it is known that OPN not only contributes to the preparation of bone matrix deposition, but also has a major role in the inflammatory response and hypoxia. Thus, when considering the present results, it is possible that early gene responses are driven by oxygen delivery and later gene responses are driven by matrix modeling responses, suggesting that different mechanisms can drive OPN gene expression in 3D cultures.

Mechanical stimulation has been shown to increase bone production *in vivo* as well as osteoblast activity *in vitro*. Here, we believe that the increase in infiltration and coverage are not due to mechanical stimulation, but rather to the increase in oxygen and nutrient delivery as well as the removal of waste. Supporting evidence implies that mass transport can mediate osteoblast activity (28), and that the shear stresses experienced by osteoblasts in the present study only reached 0.00017 Pa, which is several orders of magnitude lower than those required for cellular response to mechanical stimulation (29). Furthermore, previous research in our lab showed that mechanical stimulation (fluid-induced shear stress – 2.2 Pa) did not affect the gene expression of VEGF expression (30).

The goal of this study was to link the effects of perfusion flow on early-response gene expression to long-term cellular survival and infiltration within 3D tissue engineering scaffolds. Our bioreactor system increased relative cell number as well as cellular infiltration in 3D scaffolds. Furthermore, gene expression results suggest that perfusion flow reduces hypoxic-like conditions in our system. As hypoxia contributes to the control of bone tissue structure, understanding the molecular mechanisms behind

hypoxic gene expression has important indications for successful bone tissue engineering. Thus, the consideration of hypoxia in 3D perfusion bioreactors will advance our progress in bone tissue engineering.

Acknowledgments

This publication was made possible through funding received from the National Science Foundation Graduate Research Fellowship Program and the Michigan Space Grant Consortium.

References

1. Yaszemski MJ, Payne RG, Hayes WC, Langer R, Mikos AG 1996 Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. Biomaterials **17**(2)**:**175-85.

2. Bauer TW 2007 An overview of the histology of skeletal substitute materials. Arch Pathol Lab Med **131**(2)**:**217-24.

3. Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA 2007 Cell-Based Bone Tissue Engineering. PLoS Med **4**(2)**:**e9.

4. Volkmer E, Drosse I, Otto S, Stangelmayer A, Stengele M, Kallukalam BC, Mutschler W, Schieker M 2008 Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. Tissue Eng Part A **14**(8)**:**1331-40.

5. Malda J, Klein TJ, Upton Z 2007 The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng **13**(9)**:**2153-62.

6. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE 2003 Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng **9**(6)**:**1197-203.

7. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG 2002 Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A **99**(20)**:**12600-5.

8. Jaasma MJ, O'Brien FJ 2008 Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A **14**(7)**:**1213-23.

9. Du D, Furukawa KS, Ushida T 2009 3D culture of osteoblast-like cells by unidirectional or oscillatory flow for bone tissue engineering. Biotechnol Bioeng **102**(6)**:**1670-8.

10. Li D, Tang T, Lu J, Dai K 2009 Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. Tissue Eng Part A **15**(10)**:**2773-83.

11. Holtorf HL, Jansen JA, Mikos AG 2006 Modulation of cell differentiation in bone tissue engineering constructs cultured in a bioreactor. Adv Exp Med Biol **585:**225-41.

12. Vance J, Galley S, Liu DF, Donahue SW 2005 Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release. Tissue Eng **11**(11-12)**:**1832-9.

13. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG 2003 Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci U S A **100**(25)**:**14683-8.

14. Wang Y, Uemura T, Dong J, Kojima H, Tanaka J, Tateishi T 2003 Application of perfusion culture system improves in vitro and in vivo osteogenesis of bone marrow-derived osteoblastic cells in porous ceramic materials. Tissue Eng **9**(6)**:**1205-14.

15. Plunkett NA, Partap S, O'Brien FJ Osteoblast response to rest periods during bioreactor culture of collagen-glycosaminoglycan scaffolds. Tissue Eng Part A **16**(3)**:**943-51.

16. Zhao F, Chella R, Ma T 2007 Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: Experiments and hydrodynamic modeling. Biotechnol Bioeng **96**(3)**:**584-95.

17. Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG 2006 In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci U S A **103**(8)**:**2488-93.

18. Holtorf HL, Datta N, Jansen JA, Mikos AG 2005 Scaffold mesh size affects the osteoblastic differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor. J Biomed Mater Res A **74**(2)**:**171-80.

19. Potier E, Ferreira E, Andriamanalijaona R, Pujol JP, Oudina K, Logeart-Avramoglou D, Petite H 2007 Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone **40**(4)**:**1078-87.

20. Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J, Arnett TR 2006 Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. Exp Cell Res **312**(10)**:**1693-702.

21. D'Ippolito G, Diabira S, Howard GA, Roos BA, Schiller PC 2006 Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone **39**(3)**:**513-22.

22. Park JH, Park BH, Kim HK, Park TS, Baek HS 2002 Hypoxia decreases Runx2/Cbfa1 expression in human osteoblast-like cells. Mol Cell Endocrinol **192**(1-2)**:**197-203.

23. Cowin SC 2001 Bone mechanics handbook, 2nd ed. CRC Press, Boca Raton, FL, pp 1 v. (various pagings).

24. Cornell CN 1999 Osteoconductive materials and their role as substitutes for autogenous bone grafts. Orthop Clin North Am **30**(4)**:**591-8.

25. Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29**(9)**:**e45.

26. Steinbrech DS, Mehrara BJ, Saadeh PB, Chin G, Dudziak ME, Gerrets RP, Gittes GK, Longaker MT 1999 Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro. Plast Reconstr Surg **104**(3)**:**738-47.

27. Lee CM, Genetos DC, You Z, Yellowley CE 2007 Hypoxia regulates PGE(2) release and EP1 receptor expression in osteoblastic cells. J Cell Physiol **212**(1)**:**182-8.

28. Donahue TL, Haut TR, Yellowley CE, Donahue HJ, Jacobs CR 2003 Mechanosensitivity of bone cells to oscillating fluid flow induced shear stress may be modulated by chemotransport. J Biomech **36**(9)**:**1363-71.

29. Jungreuthmayer C, Donahue SW, Jaasma MJ, Al-Munajjed AA, Zanghellini J, Kelly DJ, O'Brien FJ 2009 A comparative study of shear stresses in collagen-glycosaminoglycan and calcium phosphate scaffolds in bone tissue-engineering bioreactors. Tissue Eng Part A **15**(5)**:**1141-9.

30. Barron M 2010 Mechanical Stimulation Mediates Gene Expression in MC3T3 Osteoblastic Cells Differently in 2D and 3D Environments. Journal of Biomechanical Engineering **132**.

CHAPTER FIVE
2D Co-cultures

Introduction

The importance of blood vessels to bone development and fracture repair has been known for centuries (1,2). Proper vascularization is critical for bone formation, bone growth, and fracture healing (1-4),(5), providing bone tissue with oxygen, nutrients, and growth factors, as well as the removing waste products. In addition to bone growth and repair, vascularization is vital during implant assimilation (6-9). As tissue-engineering bone is emerging as a potential source of tissue implants, vascularization must be considered.

One of the major limitations of tissue-engineered bone is that once implanted *in vivo*, significant amounts of time are required for host vasculature to infiltrate the scaffold. Current methods rely on this in-growth of vasculature from host tissue (6,8), which can lead to poor vascular in-growth (6). To address this, research has been focused on the formation of a vascular network within tissue-engineered bone (6,8-11). This can be accomplished by co-culturing bone cells and endothelial cells, which are important components of the vasculature.

Co-culturing bone cells and endothelial cells has shown some success (6-12), with the formation of vascular-like networks in vitro (8-10), as well as the up-regulation of bone-specific alkaline phosphatase (7,10,13,14). Literature also reports that endothelial cells can enhance the proliferation and differentiation of bone marrow-derived fibroblasts (6).

As previous research suggests the potential benefits of co-culturing osteoblasts and endothelial cells, we attempted to determine an appropriate co-culturing ratio to utilize in three-dimensional (3D) cultures. We hypothesized that co-culturing endothelial cells at a low percentage (with osteoblasts), would increase osteoblast proliferation as well as osteoblast-specific gene expression.

Materials and Methods

Cell Culture

Mouse osteoblast-like cells (MC3T3-E1 subclone 4, ATCC, Manassas, VA) were cultured in alpha-MEM (Invitrogen, Carlsband, CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Mouse microvascular endothelial cells (EC) (EOMA, ATCC, Manassas, VA) were cultured in dulbeccos-MEM (Invitrogen, Carlsband, CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Once cells reached ~70% confluency, they were trypsinized, re-suspended in culture medium, and statically seeded into 6-well culture plates. Samples were then incubated for 24 hours before experimentation. During experimentation, osteoblast differentiation media was used for all experimental treatments (control samples were not differentiated) by adding 10 mM beta-glycerol phosphate and 50 mg/mL ascorbic acid to alpha-MEM with 10% FBS and 1% pen/strep. 24-well culture dishes were seeded at a density of 30,000 cells (MC3T3 and EOMA combined) per well with the following four endothelial cell percentages: 25%, 10%, 5%, 0%. A control treatment was also included, which consisted of osteoblasts alone cultured with non-differentiation culture media. Following ten days of static culture, samples were processed to determine either total cell population (n=3) or endothelial cell coverage (n=3).

Cell Population

After ten days of culture, samples were trypsinized and re-suspended in culture medium. 10 µl of the cell suspension was added to 10 µl of trypan blue, and a haemocytometer was utilized to determine cell number. Standard calculations were uses to determine total cell number for each sample.

Endothelial cell coverage

Following ten days of culture, samples were washed with PBS, biotin-conjugated antibody (1:250 anti-mouse CD31 – Endothelial Cell specific) was added, and samples were incubated for 1 hour. Samples were again washed and the secondary antibody (1:500, Alexa Fluor 755) was added and samples were incubated for 45 minutes. Three

images were captured from each sample (covering ~50% of the section) from each sample at 100x total magnification using both brightfield and fluorescent microscopy. The total cell area was calculated by outlining the entire cell area (made easier as plates were over-confluent) in Bioquant. Endothelial cell area was also calculated by outlining the "nodes" of endothelial cells (Figure 16) in Bioquant. Endothelial cell coverage was calculated by dividing the area of endothelial cell coverage by the total area of cell coverage.

Gene Expression

Osteoblasts and endothelial cells were co-cultured at 2% endothelial cells to determine whether co-cultures affect osteoblast-specific gene expression. Following 14 days of static culture, RNA was isolated from samples using the Promega SV total RNA Isolation kit according to manufacturer's instructions. RNA concentration and quality was determined using spectrophotometer (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, Rockland, DE) readings at 260, 230, and 280 nm. Gel electrophoresis was used to verify RNA integrity. RNA was reverse transcribed into cDNA using a reaction mix consisting of Superscript II reverse transcriptase (Invitrogen, Calrsbad, CA), 1x first strand buffer (Invitrogen), 800 µM dNTPs (Promega), Rnase out recombinant ribonuclease inhibitor (Invitrogen), 48 mM dithiothreitol (Invitrogen) and 0.5 µg Oligo(dT)12-18 primer at 42°C for 20 minutes, 50°C for 10 minutes and 42°C for 1 hour in the Mastercycler Gradient Thermocycler (Eppendorf, Westbury, NY). cDNA was then used for real time PCR for alkaline phosphatase (ALP), osteocalcin (OCN), and a housekeeping gene.

Statistics

A one-factor ANOVA was used to compare total cell count (n=6) and mRNA levels (n=3) of osteoblast-specific genes. JMP IN 5.1 statistical software package (SAS, Cary, NC) was used for all statistical analyses

Results

Cell Population

Following 10 days of culture, osteoblasts alone (0% endothelial cells) cultured in differentiation media increased from an initial 30,000 cells to a final cell number of 136,000. Adding 5% endothelial cells up-regulated final cell number by 85% over osteoblasts alone ($p = 0.001$). Adding 10% endothelial cells up-regulated the final cell number by 135% over osteoblasts alone ($p = < 0.0001$). Adding 25% endothelial cells increased final cell number by 90% ($p = 0.0006$). A summary of the percentage study can be seen in Figure 14.

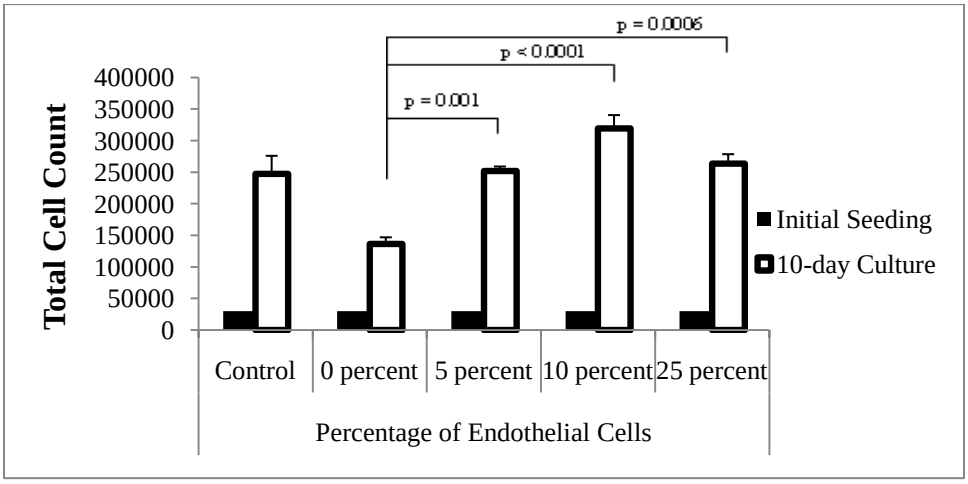

Figure 14 - Increasing the ratio of endothelial cells increases the final total cell count in osteoblast/endothelial cell co-cultures.

Endothelial Cell Coverage

The proliferation rate of endothelial cells was much higher than that of osteoblasts. This is evidenced by an increase in the level of endothelial cell coverage over the course of 10 days. For example, at an initial seeding ratio of 25% endothelial cells, 10 days of culture produced an endothelial cell coverage area of 80% (Figure 15). This suggests that the increase in cell number seen with increasing ratios of endothelial cells (Figure 14) can most likely be attributed to the proliferation of endothelial cells rather than osteoblasts. This is evident in images taken from 25% endothelial cell co-

cultures, where osteoblasts appear to have overwhelmed the surface area of the plate (Figure 16).

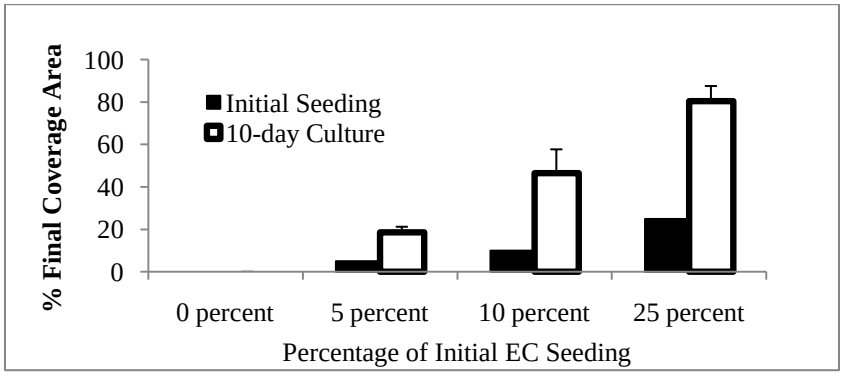

Figure 15 - Increasing the ratio of endothelial cells causes endothelial cells to overwhelm the total cell area.

Qualitatively, it was observed that at 5% co-culture, endothelial cells tended to form "nodes" of cells rather than a network of individual or small groups of cells. As the ratio of endothelial cells increased, it appeared that network formation between "nodes" was more apparent (Figure 16).

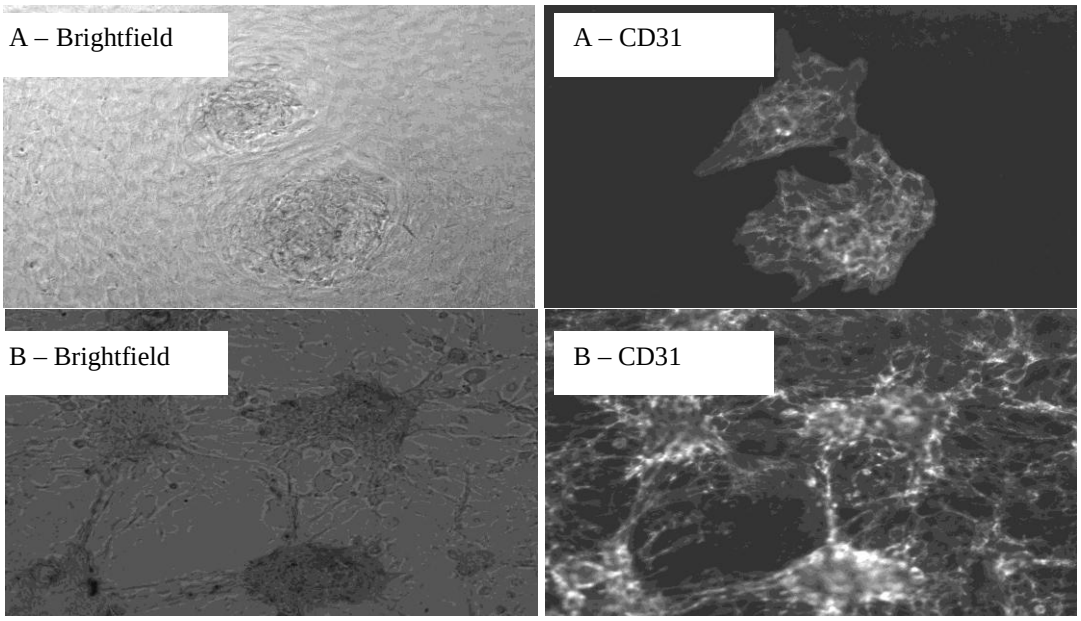

Figure 16 – A) At low ratios (5%), endothelial cells tend to form "nodes" rather than networks of individual cells. B) At higher ratios (25%), endothelial cells tend to overwhelm the cell area after 10 days of culture, but tend to form a netork between groups of cells. CD31 is only expressed by endothelial cells. 100x magnification.

Gene Expression

Alkaline phosphatase (ALP) mRNA levels were up-regulated ($p < 0.0001$) by ~130% in co-cultures after 14 days of two-dimensional culture (Figure 17). This is consistent with results seen in literature (7,10,13,14). In contrast, osteocalcin (OCN) mRNA levels were down-regulated ($p = 0.007$) by ~63% (Figure 17).

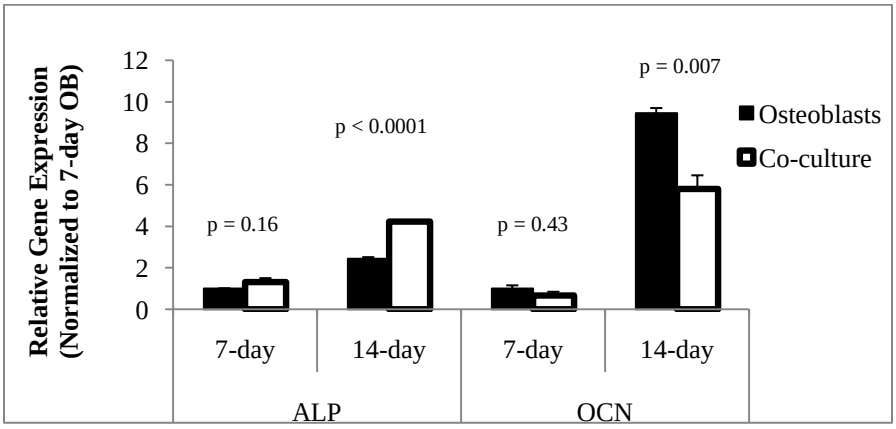

Figure 17 - ALP is up-regulated and OCN is down-regulated in osteoblasts co-cultured with 2% endothelial cells.

Discussion

This co-culture model included cell lines that have not been studied extensively, making it necessary to characterize an appropriate co-culture percentage before designing major studies. Co-cultures have been shown to not only increase the proliferation of osteoblasts, but also to increase the level of bone-specific ALP (7,10,13,14). Thus, the goals of this study were to determine the effects that varying ratios of endothelial cells to osteoblasts has on the proliferation and bone-specific gene expression of osteoblasts.

Cell number results suggested that higher ratios of endothelial cells cause an increase in overall cell number. However, it is clear from cell coverage results that these endothelial cells proliferate much more quickly than the osteoblasts. When co-cultured at 25%, endothelial cells rapidly proliferated, eventually covering over 80% of the total surface area by the end of 10 days. To avoid long-term studies in which endothelial cells completely overwhelm osteoblast response, a low ratio of endothelial cells was chosen

for further study. Thus, a 2% co-culture was utilized to test osteoblast-specific gene expression.

Osteocalcin (a marker of late-stage osteoblast differentiation) gene expression was decreased in osteoblast/endothelial cell co-cultures. While initially concerning, the decrease in OCN may be explained by a prolonged state of proliferation in osteoblasts co-cultured with endothelial cells. This has been seen in some studies (15,16), and would explain reductions in late-stage markers of osteoblast differentiation as well as increases in cell number associated with co-cultures. The increase in ALP would still be relevant, as it is an early-stage marker of differentiation that peaks in expression well in advance of genes such as OCN. If true, one would expect a subsequent peak of OCN to be several days later than would normally be expected (17). Future work should include a timecourse of bone-specific gene expression with co-cultures of osteoblasts and endothelial cells.

Ultimately, the goal is to produce bone matrix in a 3D construct. An up-regulation in ALP with co-cultures was encouraging, and supported the continued use of our co-culture model. Moving forward, we chose to use a 2% co-culture in three dimensions to continue our tissue-engineering work. Although two-dimensional results do not necessarily predict 3D results (18), expanding this co-culture model into three dimensional constructs has the potential to advance the field of bone tissue engineering.

References

1. Carano RA, Filvaroff EH 2003 Angiogenesis and bone repair. Drug Discov Today **8**(21)**:**980-9.

2. Glowacki J 1998 Angiogenesis in fracture repair. Clin Orthop Relat Res (355 Suppl)**:**S82-9.

3. John P. Bilezikian LGR, Gideon A. Rodan 2002 Principles of Bone Biology. In: Bilezikian R, Rodan (ed.) Principles of Bone Biology, 2nd ed., vol. 1 and 2. Academic Press, San Diego, pp 1700.

4. Burkhardt R, Kettner G, Bohm W, Schmidmeier M, Schlag R, Frisch B, Mallmann B, Eisenmenger W, Gilg T 1987 Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. Bone **8**(3)**:**157-64.

5. Hausman MR, Schaffler MB, Majeska RJ 2001 Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone **29**(6)**:**560-4.

6. Choong CS, Hutmacher DW, Triffitt JT 2006 Co-culture of Bone Marrow Fibroblasts and Endothelial Cells on Modified Polycaprolactone Substrates for Enhanced Potentials in Bone Tissue Engineering. Tissue Eng.

7. Stahl A, Wenger A, Weber H, Stark GB, Augustin HG, Finkenzeller G 2004 Bi-directional cell contact-dependent regulation of gene expression between endothelial cells and osteoblasts in a three-dimensional spheroidal coculture model. Biochem Biophys Res Commun **322**(2)**:**684-92.

8. Wenger A, Kowalewski N, Stahl A, Mehlhorn AT, Schmal H, Stark GB, Finkenzeller G 2005 Development and characterization of a spheroidal coculture model of endothelial cells and fibroblasts for improving angiogenesis in tissue engineering. Cells Tissues Organs **181**(2)**:**80-8.

9. Unger RE, Sartoris A, Peters K, Motta A, Migliaresi C, Kunkel M, Bulnheim U, Rychly J, Kirkpatrick CJ 2007 Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials. Biomaterials **28**(27)**:**3965-76.

10. Rouwkema J, de Boer J, Van Blitterswijk CA 2006 Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. Tissue Eng **12**(9)**:**2685-93.

11. Villars F, Guillotin B, Amedee T, Dutoya S, Bordenave L, Bareille R, Amedee J 2002 Effect of HUVEC on human osteoprogenitor cell differentiation needs

heterotypic gap junction communication. Am J Physiol Cell Physiol **282**(4)**:**C775-85.

12. Akita S, Tamai N, Myoui A, Nishikawa M, Kaito T, Takaoka K, Yoshikawa H 2004 Capillary vessel network integration by inserting a vascular pedicle enhances bone formation in tissue-engineered bone using interconnected porous hydroxyapatite ceramics. Tissue Eng **10**(5-6)**:**789-95.

13. Villars F, Bordenave L, Bareille R, Amedee J 2000 Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? J Cell Biochem **79**(4)**:**672-85.

14. Clarkin CE, Emery RJ, Pitsillides AA, Wheeler-Jones CP 2008 Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated crosstalk to endothelial cells. J Cell Physiol **214**(2)**:**537-44.

15. Clarkin CE, Garonna E, Pitsillides AA, Wheeler-Jones CP 2008 Heterotypic contact reveals a COX-2-mediated suppression of osteoblast differentiation by endothelial cells: A negative modulatory role for prostanoids in VEGF-mediated cell: cell communication? Exp Cell Res **314**(17)**:**3152-61.

16. Meury T, Verrier S, Alini M 2006 Human endothelial cells inhibit BMSC differentiation into mature osteoblasts in vitro by interfering with osterix expression. J Cell Biochem **98**(4)**:**992-1006.

17. Stein GS, Lian JB 1993 Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev **14**(4)**:**424-42.

18. Barron M 2010 Mechanical Stimulation Mediates Gene Expression in MC3T3 Osteoblastic Cells Differently in 2D and 3D Environments. Journal of Biomechanical Engineering **132**.

CHAPTER SIX
3D Co-cultures

Perfusion flow enhances the infiltration of osteoblasts and endothelial cells into 3D calcium phosphate scaffolds.

Matthew J. Barron, B.S., Jeremy Goldman, Ph.D., Seth W. Donahue, Ph.D.

Introduction

While autografts are currently the best available option for bone grafting, the pain and morbidity associated with second surgeries combined with the limited availability of autograft sources highlights the need for alternative grafting materials (1-3). An ideal bone graft provides scaffolding for new bone tissue to grow into (osteoconductive), induces host tissue to produce new bone matrix (osteoinductive), and supplies a cellular source for new tissue growth (osteogenic) (3-6). Although 3D bone tissue engineering can provide all three traits, one of the biggest limitations to overcome is the maintenance of cellular viability within the center of 3D constructs upon *in vivo* implantation (7-11).

Proper vascularization is critical for successful bone growth and development, and is one of the first phases seen during fracture healing (11-16). Without proper vascularization, bone tissue would face limited growth and hypoxia (13). As the vasculature provides bone tissue with oxygen, nutrients, growth factors, and the removal of waste, it is not surprising that vascularization is also essential for successful implant integration. Currently, implanted bone grafts rely on the in-growth of existing vessels from host tissue (7,9), which can take significant amounts of time, leading to poor vascularization and implant rejection (7). One solution to this clinical problem is to introduce tissue-engineered bone is pre-vascularized by a rudimentary vascular system (7,9,10,17,18). A common approach to this solution is the co-culture of bone cells and endothelial cells on three-dimensional constructs.

There has been some success with co-culturing bone cells and endothelial cells to overcome the lack of vasculature in tissue-engineered bone (7-10,17-19). These studies have shown that not only will endothelial cells form a pre-vascular network during co-

culture (9,10,17), but also that endothelial cells can augment osteoblastic activity when both cell types are in direct contact (8,9,17,20,21). Additionally, recent work has shown that 3D co-cultures will integrate with host tissue *in vivo*, with the formation of an improved vascular network upon implantation (22-24).

Although static co-cultures have had some success, it is not known how a co-culture model will respond to a dynamic environment. Perfusion bioreactors actively deliver culture medium to cell-seeded porous scaffolds through pump-driven fluid flow. Perfusion flow increases cellular viability and oxygen transport within osteoblast-seeded 3D constructs (25-30), leading to enhanced gene expression (25,27,31), protein production (26,29,32-35), and calcium deposition (26,33,36-38). In addition, fluid flow also activates endothelial cells, increasing angiogenic gene expression and the formation of tube-like structures (12,20,39-42). Taken together, these results suggest that the use of a perfusion bioreactor with osteoblast/endothelial cell co-cultures may accelerate the vascularization of tissue-engineered bone.

We hypothesize that co-culturing osteoblasts and endothelial cells will increase osteogenic and angiogenic gene expression in 3D calcium phosphate scaffolds. Furthermore, perfusion flow will increase the length of endothelial cell aggregations in 3D porous scaffolds. This work will help elucidate the contributions of endothelial cells to osteoblastic gene activity, as well as the effects that a dynamic environment has on the formation of a vascular network in co-cultures.

Materials and Methods

Cell Culture

Mouse osteoblast-like cells (MC3T3-E1 subclone 4, ATCC, Manassas, VA) were cultured in alpha-MEM (Invitrogen, Carlsband, CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Mouse microvascular endothelial cells (EC) (EOMA, ATCC, Manassas, VA) were cultured in dulbeccos-MEM (Invitrogen, Carlsband, CA) with 10% FBS (Hyclone, Logan, UT) and 1% pen/strep (Cellgro, Herndon, VA). Once cells reached ~70% confluency, they were trypsinized, re-suspended in culture medium, and statically seeded onto 3D calcium phosphate scaffolds

(1,000,000 cells/scaffold; 98% osteoblasts, 2% endothelial cells) (BD Biosciences, San Jose, CA). The scaffolds have an interconnected porosity of ~ 60% with an average pore size of 200-400 microns. This is comparable to trabecular bone (50-90% porosity, 500-1500 micron pore size (43)), and within the range suggested to be optimal for bone regeneration (150 – 650 microns) in porous scaffolds (44). Samples were then incubated for 1 hour to allow cell adhesion, covered with media, and incubated for 24 hours before experimentation. During experimentation, osteoblast differentiation media was used by adding 10 mM beta-glycerol phosphate and 50 mg/mL ascorbic acid.

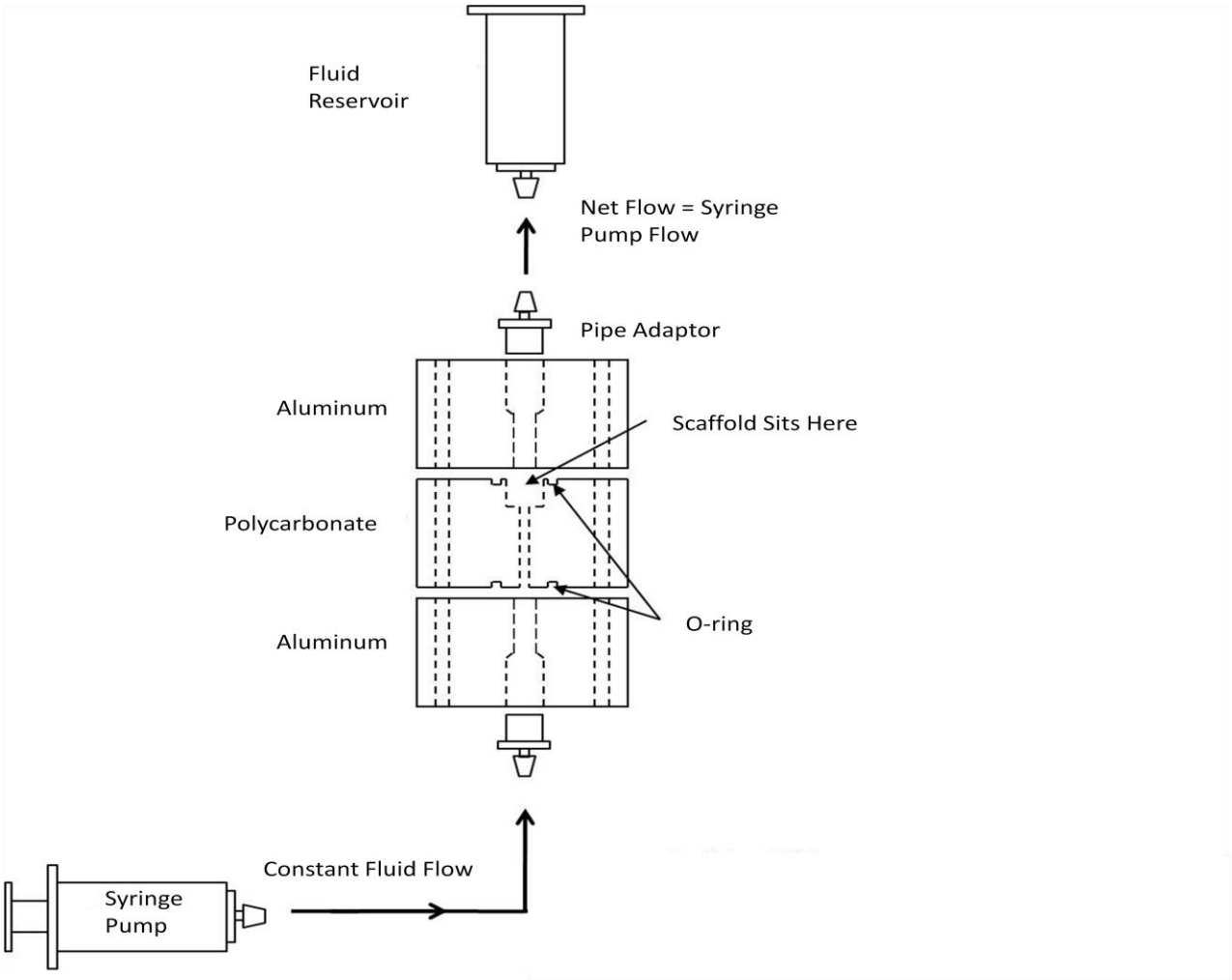

Figure 18 - Bioreactor setup with only the syringe pump used for long-term perfusion incubation. The linear actuator was not used for high-shear oscillatory fluid flow. See text for more detail.

Experimental Design

Four treatment groups were tested: two cell populations (osteoblasts alone and osteoblast/endothelial cell co-culture) with two culture methods (static culture and perfusion flow). Static samples were cultured in 24-well plates, while perfusion samples were cultured in bioreactor chambers. All samples remained incubated at 37°C with 5% CO_2 for the duration of the experiment. Flow was initiated immediately after samples were placed into the chambers and continued for the duration of the experiment. After 7 or 14 days of culture, samples were removed from static or perfusion incubation and RNA was isolated, or samples were fixed in 10% buffered formalin for histological processing. Gene expression was assessed after both time points (n=3 per group), and histological analysis performed at 14 days (n=3 per group).

Perfusion Flow Bioreactor

A custom perfusion bioreactor was used to deliver culture media to osteoblasts on calcium phosphate scaffolds. Each bioreactor chamber consists of a top and bottom aluminum block, between which a polycarbonate block is secured (Figure 18). A 6-mm hole was drilled out of the polycarbonate block to fit a porous calcium phosphate scaffold 5-mm in diameter and 3.5-mm in height (BD Biosciences, Bedford, MA). A 4-mm hole was drilled through the center of all three blocks to allow fluid flow through the scaffold. Grooves were machined around both the hole and the space on each side of the polycarbonate block to fit a #11 Viton O-ring (Allorings, Hampton Falls, NH). A $1/8^{th}$ inch barbed pipe connector was threaded into both the top and bottom aluminum blocks and connected to $1/8^{th}$ inch silicone tubing (Harvard Apparatus, Holliston, MA). The silicone tubing is permeable to CO_2 and O_2, permitting adequate gas exchange. Tubing connected the top of the chamber to a 140 ml syringe fluid reservoir, and the bottom of the chamber to syringe pump (Harvard Apparatus). The pump delivered media at a rate of 0.075 ml/min, with flow being reversed every 24 hours. Media was replenished every third day.

RNA Isolation and RT-PCR

After 7 and 14 days of culture, cell-seeded scaffolds were placed in 2 ml tubes and crushed in SV Lysis Buffer with a glass rod. After lysis, dilution buffer was added along with 200 mM phosphate buffer to elute the RNA. RNA was isolated from cells using the Promega SV total RNA Isolation kit according to manufacturer's instructions. RNA concentration and quality was determined using spectrophotometer (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, Rockland, DE) readings at 260, 230, and 280 nm. Gel electrophoresis was used to verify RNA integrity. RNA was reverse transcribed into cDNA using a reaction mix consisting of Superscript II reverse transcriptase (Invitrogen, Calrsbad, CA), 1x first strand buffer (Invitrogen), 800 µM dNTPs (Promega), Rnase out recombinant ribonuclease inhibitor (Invitrogen), 48 mM dithiothreitol (Invitrogen) and 0.5 µg Oligo(dT)$_{12\text{-}18}$ primer at 42°C for 20 minutes, 50°C for 10 minutes and 42°C for 1 hour in the Mastercycler Gradient Thermocycler (Eppendorf, Westbury, NY). cDNA was then used for real time PCR for the genes of interest and the housekeeping genes (Table 8).

All reactions were performed in the StepOnePlus real-time PCR system (Applied Biosystems) under the following cycle parameters: hot start at 95°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds. Threshold fluorescence was set to 1500 dR and the C_t value was determined for all reactions. C_t values were used to determine the relative up or down regulation for each gene using the relative standard curve method and normalizing to housekeeping genes that do not change between treatments (45).

Histological Analysis

Preparation

Following fixation, 14-day samples were placed in cassettes, covered in OCT freeze medium, and placed under vacuum for 48 hours to remove air bubbles. Samples were then removed, flash frozen, and sectioned on a soft tissue microtome (Microm International, Waldorf Germany) at a thickness of 10 microns. Sections were taken from mid-way through the scaffold running parallel to the direction of perfusion flow (Figure 19). Sections were decalcified in 0.5% EDTA for 20 minutes. After washing with PBS,

Gene Name	Function	Primer Sequence
Osteocalcin	Osteoblast Differentiation	F: 5'- GAGTCTGACAAAGCCTTCATGTCC -3'; R: 5'- TGATAGCTCGTCACAAGCAGGGTTA -3'
Osteopontin	Osteoblast Differentiation	F: 5'- CAGCTGGATGAACCAAGTCTGGAA -3'; R: 5'- ACTAGCTTGTCCTTGTGGCTGTGA -3'
Alkaline Phosphatase	Osteoblast Differentiation	F: 5'-GCCCTCTCCAAGACATATA-3'; R: 5'-CCATGATCACGTCGATATCC-3'
RUNX-2	Osteoblast Differentiation	F: 5'- AGAGTCAGATTACAGATCCCAGGC -3'; R: 5'- GTCAGAGGTGGCAGTGTCATCAT -3'
Collagen Type I	Matrix Protein	F: 5'- TGGTTTGGAGAGAGCATGACCGAT -3'; R: 5'- TGTAGGCTACGCTGTTCTTGCAGT -3'
Vascular Endothelial Growth Factor A	Angiogenesis	F: 5'- ACAGAAGGAGAGCAGAAGTCCCAT -3'; R: 5'- ATGTGCTGGCTTTGGTGAGGTTTG -3'
VEGF Receptor 2	Angiogenesis	5-CTC TGT GGG TTT GCC TGG CGA TTT TCT-3; 5-GGG GAT CAC CAC AGT TTT GTT CTT GTT-3
Cyclophillin	Housekeeping	F: 5'- TCATGTGCCAGGGTGGTGACTTTA; R: 5'- ATGCTTGCCATCCAGCCATTCAGT -3'
Beta-Actin	Housekeeping	F: 5'- ATCACTATTGGCAACGAGCGGTTC -3'; R: 5'- TCTCCTTCTGCATCCTGTCAGCAA -3'
Ubiquitin	Housekeeping	F: 5'- CGTCGAGCCCAGTGTTACCACCAAGAAGG -3; R: 5'- CCCCCATCACACCCAAGAACAAGCACAAG -3'

Table 8 - Primer sequences and roles for genes measured in 3D co-culture study.

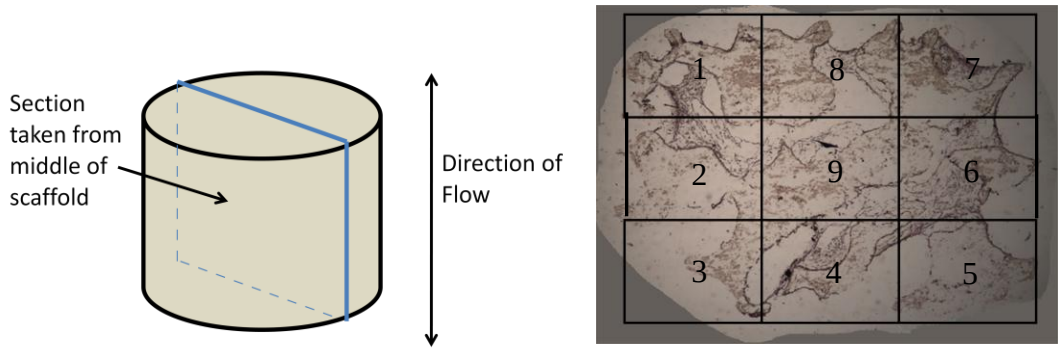

Figure 19 - Sections were removed from the middle of the scaffold parallel to the direction of flow. Nine sample regions from each section were used to quantify cell number, surface coverage, and cell/matrix area coverage. Regions 1-8 are combined and are referred to as the scaffold perimeter. Region 9 is referred to as the scaffold center.

biotin-conjugated antibody (1:250 anti-mouse CD31 – Endothelial Cell specific) was added and samples were incubated for 1 hour. Sections were again washed and the secondary antibody (1:500, Alexa Fluor 755) was added and samples were incubated for 45 minutes. Following a brief rinse, sections were also stained with hematoxylin and eosin. Prior to fluorescent microscopy, DAPI was added to the sections to identify cell nuclei. Images were captured for the entire section from each sample at 100x total magnification using both brightfield and fluorescent microscopy. Nine images were taken in total, three from the upper, middle, and lower thirds of each section (Figure 19). Fluorescent images were used to distinguish between cell types, as DAPI identifies all cell nuclei and CD31 stains membrane receptors on endothelial cells. Brightfield images were used to determine total cell number, surface coverage, and cell/matrix area coverage.

Cell Number, Surface Coverage, Cell/Matrix Area Coverage

Cell number, surface coverage, and cell/matrix area coverage were determined around the perimeter of the scaffold as well as in the scaffold center, allowing for the assessment of whether perfusion flow is more effective in areas of poor diffusion. For the scaffold perimeter, regions 1-8 were combined. For the scaffold center, only the center image (region 9) was used for measurement (Figure 19).

Average cell number was determined by counting the total cell number in each region within a section. In the scaffold perimeter, cell number was averaged for all 8 regions (Figure 19) for easier comparison with the scaffold center (region 9 – Figure 19).

Surface coverage (represented by % total perimeter) was calculated by measuring the ratio of cell coverage around the surface area of scaffold pores to the total surface area of scaffold pores (including outer surface) using Bioquant (Bioquant Osteo, Nashville, TN) software. Since surface coverage was calculated as a percentage of total coverage area, so averaging all 8 regions was not necessary to compare with the scaffold center (region 9 – Figure 19).

Cell/matrix area coverage (represented as % pore space) was established by measuring the ratio of cell and matrix area within pore spaces to total pore space using a standard threshold value in Bioquant (Figure 20). Since cell/matrix area coverage was

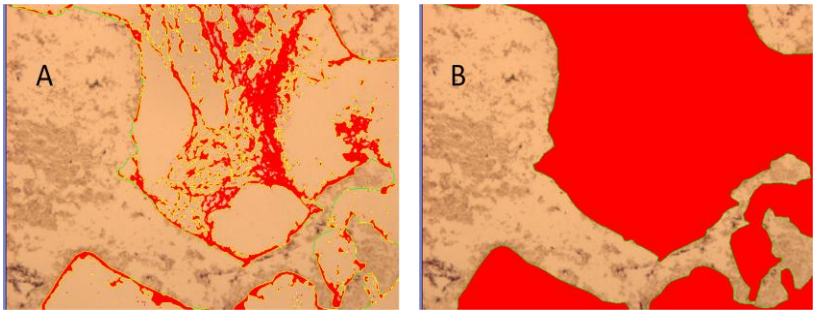

Figure 20 - To determine cell/matrix area coverage, a ratio was calculated between A) area of cell/matrix coverage and B) total area of pore spaces. The area in red was used for measurement and was determined by setting a threshold value in Bioquant.

calculated as a percentage of total coverage area, so averaging all 8 regions was not necessary to compare with the scaffold center (region 9 – Figure 19).

To measure the length of endothelial cell aggregations, fluorescent microscopy was used to distinguish between cell types, and Bioquant was used to determine endothelial cell aggregate length based on standard scale measurements (Figure 21). The number of endothelial cell aggregates was small due to the low ratio of endothelial cells seeded initially. Thus, additional sections were taken from 25% and 75% of the way through each sample for additional measurements of aggregation length.

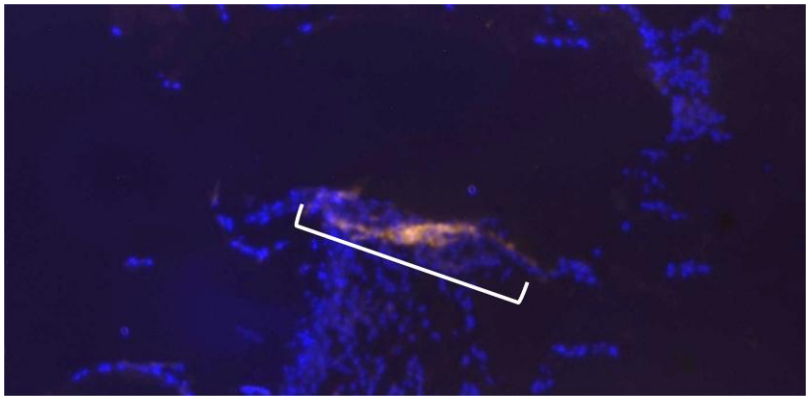

Figure 21 - Fluorescent image of osteoblast/endothelial cell co-cultures - dapi stained nuclei (Blue) and CD31 antibody (orange) for endothelial cells. White bracket designates aggregate length measurement.

Statistics

Gene Expression

Three samples were tested for each experimental condition. A two-factor ANOVA (cell population and incubation method) was used to establish model significance. Significance was established for both factors, so grouping was not possible. JMP IN 5.1 statistical software package (SAS, Cary, NC) was used for all statistical analyses.

Histological Analysis

A two-factor ANOVA (cell population and culture method) was used to establish model effects for histological endpoints. No significance was associated with cell population (osteoblast vs. co-culture) for cell number ($p = 0.94$), surface coverage ($p = 0.64$), or cell/matrix area coverage ($p = 0.43$), and no interactions existed between factors. Thus, values were combined for osteoblast and co-culture treatments. A one-factor (ANOVA) was then used to compare cell number, surface coverage, and cell/matrix area coverage between static culture and perfusion flow in both the scaffold perimeter (regions 1-8 combined) and the scaffold center (region 9) (Figure 19).

For endothelial cell aggregation length, each aggregation within a section was measured. As the lengths within each biological replicate were normally distributed and were not significantly ($p = 0.33$) different between samples within a treatment, a one-way ANOVA was run comparing *all* aggregation lengths within static culture to *all* aggregation lengths within the perfusion flow treatment. JMP IN 5.1 statistical software package (SAS, Cary, NC) was used for all statistical analyses.

Results

Gene Expression

Incubation Method - Perfusion Flow vs. Static Culture

After 7 days of culture, mRNA levels of both ALP and OCN were up-regulated (over static culture) by 2.4 fold ($p = 0.0003$) and 10.0 fold ($p = 0.0009$), respectively, when cultures (osteoblast cultures and co-cultures combined) were exposed to perfusion

flow (Table 9). This was not surprising, as ALP and OCN, both markers of osteoblast differentiation, have been shown in several instances to be increased in 3D scaffolds exposed to perfusion culture (25,26,33,37,46). RUNX-2, OPN, Col1, and VEGF did not change ($p > 0.2$).

Gene	***7-Day Fold Change***	***p-value***	***14-Day Fold Change***	***p-value***
ALP	*↑2.4*	*0.0003*	*NC*	*0.11*
OCN	*↑10.0*	*0.0009*	*NC*	*0.91*
RUNX-2	*NC*	*0.26*	*↓ 0.58*	*0.0004*
VEGF	*NC*	*0.55*	*↓ 0.85*	*0.003*

Table 9 - Changes in mRNA levels with perfusion flow. P-values represent model effects for incubation method (osteoblast cultures and co-cutlures combined).

After 14 days of culture, perfusion flow down-regulated mRNA levels of RUNX-2 and VEGF by 0.58 ($p = 0.0004$) and 0.85 fold ($p = 0.003$), respectively. These results were unexpected as both genes have been shown to be up-regulated as osteoblasts differentiate, and 7-day mRNA levels indicated an increase in differentiation-related genes (ALP and OCN).

<u>Cell Population – Osteoblast Culture vs. Endothelial Cell/Osteoblast Co-culture</u>

Co-culturing endothelial cells with osteoblasts (static and perfusion combined) caused changes in only one gene at either time point. After 14 days of culture, RUNX-2 was down-regulated in co-cultures by 0.17-fold ($p = 0.0024$) over osteoblasts alone. No other changes were evident ($p > 0.2$).

Histological Analysis – Cell Number, Surface Coverage, Cell Area Coverage

Under static culture, cell number was 60% lower ($p = 0.003$) in the scaffold center (region 9) as compared to the scaffolds' outer perimeter (regions 1-8) (Figure 22). Surface coverage and cell/matrix area coverage were not significantly different ($p > 0.45$).

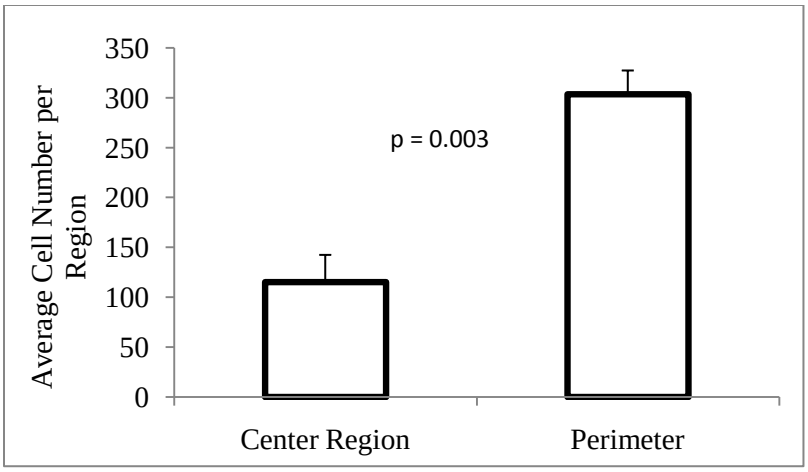

Figure 22 - Cell Number is Lower in the Middle Region of 3D Scaffolds Cultured Statically for 14 Days

Around the scaffold perimeter, perfusion flow had no effect on cell number ($p = 0.09$), surface coverage ($p = 0.58$), or cell/matrix area coverage ($p = 0.06$). However, in the scaffold center perfusion flow increased cell number ($p = 0.03$), surface coverage ($p = 0.02$), and cell/matrix area coverage ($p = 0.03$) by 220%, 84%, and 280% respectively compared to static culture (Figures 23-25). These findings suggest the scaffolds' centers are more responsive to perfusion flow than the outer perimeter. These results are consistent with both literature and our hypothesis.

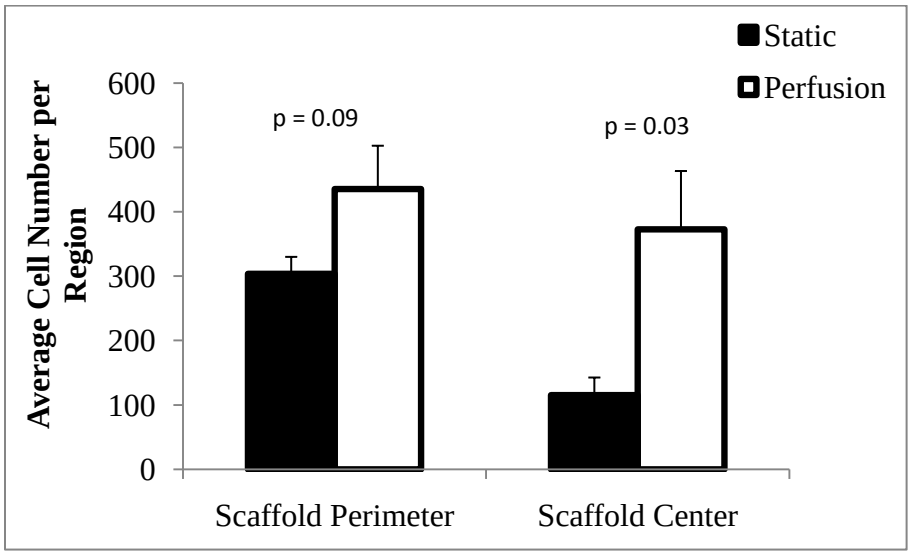

Figure 23 - Cell number is increased in 3D scaffolds when exposed to perfusion flow for 14 days

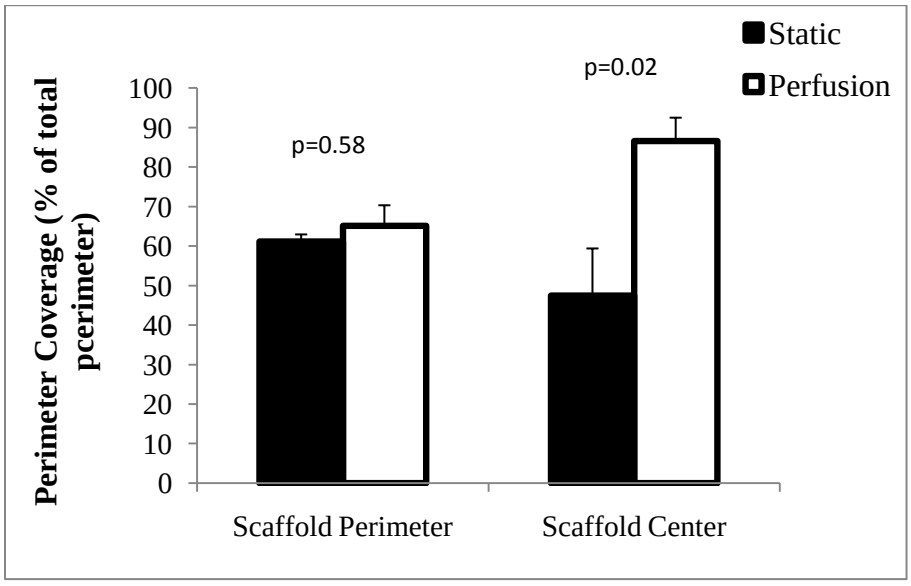

Figure 24 - Surface coverage is increased in 3D scaffolds when exposed to perfusion flow for 14 days

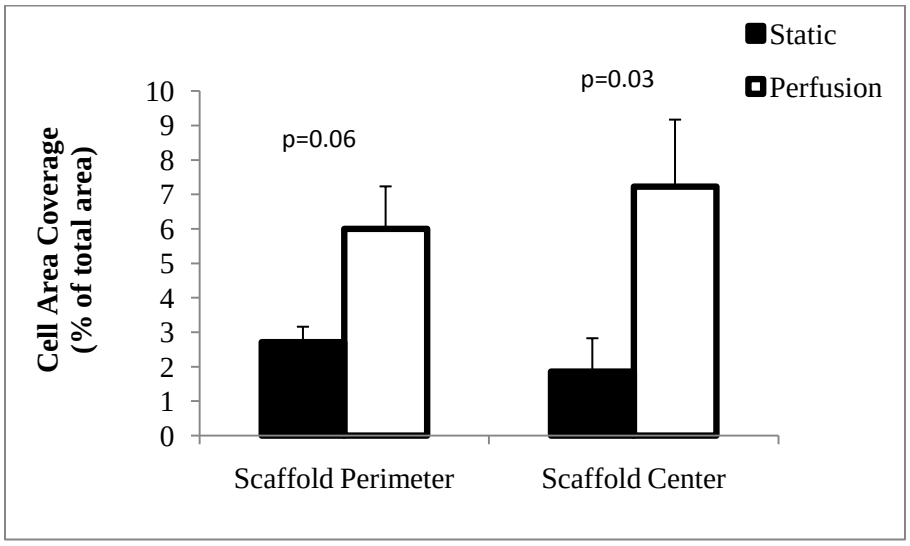

Figure 25 – Cell/matrix area coverage is increased in 3D scaffolds when exposed to perfusion flow for 14 days

Histological Analysis – Endothelial Cell Aggregate Length

Qualitative Observations

Endothelial cells were found along the surfaces of the scaffold pores rather than within the matrix formed by osteoblasts inside the pore structures. Additionally, EC aggregations were found in all 9 sample regions of the scaffold, and did not appear to be more highly concentrated in any particular region of the scaffolds. Furthermore, endothelial cells were not found as individual cells, but rather as aggregations of many cells. This was true throughout all regions. Aggregations seemed to be oriented in all directions on the scaffold surface, and did not tend to be aligned in the direction of fluid flow.

Endothelial Cell Aggregate Length

Aggregations were measured from each region of the scaffold sections. The number of aggregations in each region varied considerably, and there was no difference in the average aggregate number ($p = 0.31$) in the scaffold perimeter and the scaffold center. Furthermore, there was no difference ($p = 0.20$) in aggregate number between static culture and perfusion flow (Figure 26). However, the average endothelial cell aggregate length increased by almost 50% ($p = 0.007$) in perfusion flow samples compared to static samples (Figure 27). There was no difference in total endothelial cell aggregate length in static culture vs. perfusion flow (Figure 28).

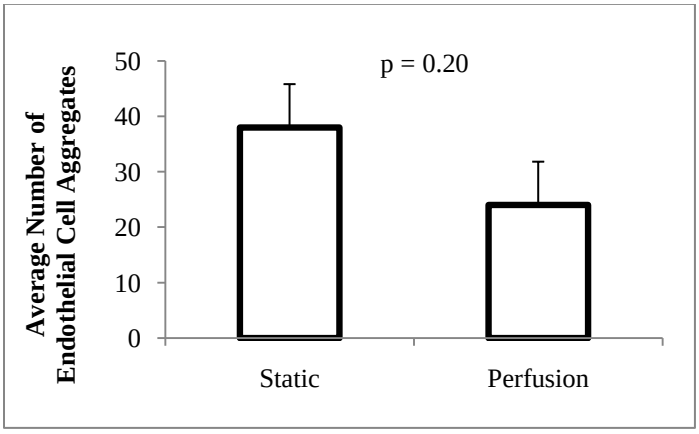

Figure 26 - The average number of endothelial cell aggregates did not change in 3D scaffolds subjected to perfusion flow compared to static conditions.

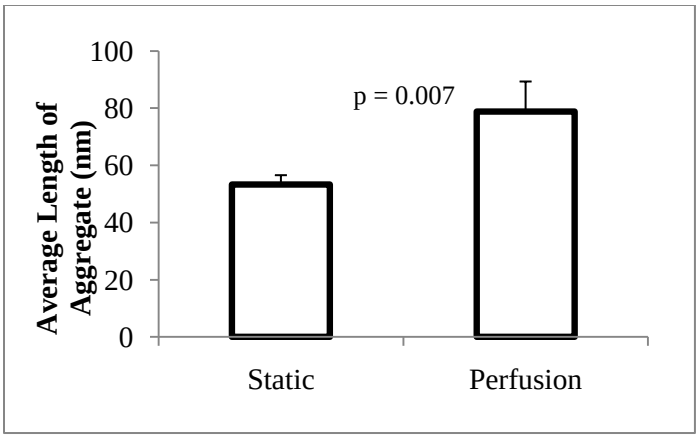

Figure 27 - The average length of endothelial cell aggregates was increased in cell-seeded 3D scaffolds and exposed to perfusion flow (n = 72 aggregates) compared to static conditions (n = 114 aggregates).

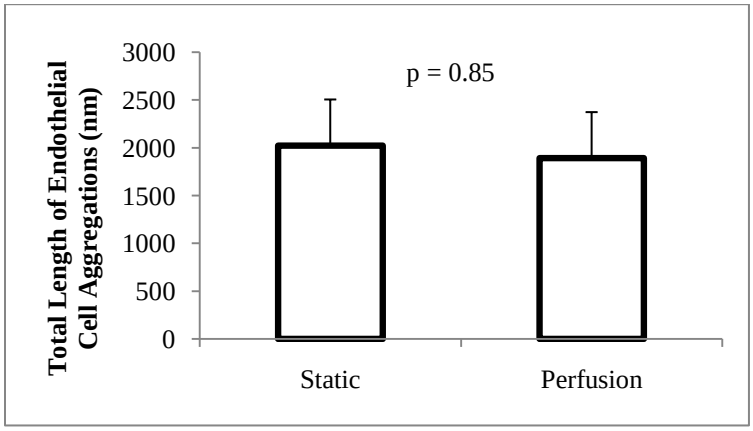

Figure 28 - Total Length of Endothelial Cell Aggregations Does Not Change With Perfusion Flow

Discussion

The ultimate goal of tissue engineering is to produce high quality bone tissue *in vitro* to be used as a clinical alternative to autografting. 3D structures cultured *in vitro* encounter limited vascular invasion upon implantation. Here, we co-cultured endothelial cells and osteoblasts to develop a method which might improve vascularization following implantation of tissue-engineered bone constructs *in vivo.* Specifically, we used perfusion flow to increase the length of endothelial cell aggregations within co-cultures

while at the same time increasing osteoblast-specific gene expression and the cell number, perimeter coverage, and cell area coverage in the center of the scaffold.

Although there is no existing literature on the effects of perfusion flow on co-cultures, there have been studies which have achieved vascular-like network formation in static osteoblast/endothelial cell co-cultures (10). While we showed that perfusion flow can increase the length of EC aggregations, we did not provide evidence of the formation of vascular-like networks among EC populations. The process of how the endothelial cells tended to aggregate within the scaffold was not established as our priority was to determine aggregation length rather than method of formation. It is possible that ECs proliferated and continued to align themselves along the perimeter of the scaffolds' pores, rather than actively migrating together. This method of formation seems likely as we did not observe any network formation between aggregations, nor did we identify any individual endothelial cells outside of major aggregations. The low ratio (2%) of endothelial cell seeding may not have allowed for communication between populations of endothelial cells, which is necessary for network formation. In the future, it may be necessary to increase the ratio of endothelial cells to increase interaction between aggregations, enhancing network formation.

In static culture, the center region of the scaffolds had lower cell number than in peripheral regions. Perfusion flow successfully increased cell number as well as scaffold perimeter coverage and cell area coverage in the scaffold center. These three responses were not seen along the scaffold perimeter. This amplified response within the center of the scaffold may be explained by considering diffusional limitations associated with 3D tissue engineering (4,30,47,48). 3D cell-seeded constructs have reduced cell numbers and cellular activity towards their centers due to poor nutrient and oxygen delivery. Perfusion flow has been used to mitigate these limitations by increasing oxygen and nutrient delivery (30), consequently increasing cell number, matrix production, and osteoblast-specific gene expression in the center of 3D constructs. Thus, it is not surprising that perfusion flow affected the scaffolds' center to a greater degree than it did the scaffolds' outer perimeter.

In attempting to develop vascular rudiments with osteoblast/endothelial cell co-cultures, we also demonstrated that co-culturing decreases RUNX-2 gene expression in

osteoblasts, suggesting that co-culture may slow the process of differentiation in osteoblasts, as has been seen in other studies (49). Despite this decrease in gene expression, there was no histological evidence of reduced cell number, perimeter coverage, or area coverage with co-culture.

Our results indicate that perfusion flow increases bone specific gene expression of ALP and OCN at seven days, which is consistent with previous reports (25,26,33). Furthermore, histological evidence suggests that osteoblast activity is increased with perfusion flow. In contrast to these results, RUNX-2 was reduced with perfusion flow after 14 days of culture. Osteoblast differentiation genes tend to follow a well-defined pattern of expression throughout the process of maturation. ALP, OCN, and OPN are all up-regulated transiently, tend to peak after 2-3 weeks, and subsequently drop in expression as osteoblasts differentiate into osteocytes. As RUNX-2 is a transcription factor responsible for osteoblast differentiation, it is not surprising that its expression precedes osteoblast-specific genes. A possible explanation for the decrease in RUNX-2 expression with perfusion flow is that it has reached its peak expression earlier in perfused samples, and has already begun its decline in expression at 14 days. Future research may include a timecourse to fully understand the expression patterns of osteoblast-specific genes.

Previous research has shown that perfusion bioreactors reduce hypoxia in 3D scaffolds (26,30,33). Hypoxia elicits a well-documented response from mesenchymal stem cells and osteoblasts, including the up-regulation of vascular endothelial growth factor (VEGF), a major mediator of both angiogenesis and hypoxia (50,51). Here, we have shown that perfusion flow reduces the expression of VEGF in osteoblast and co-cultures seeded on 3D scaffolds. In addition, cell number, scaffold perimeter, and cell area coverage are all increased in the scaffold center with perfusion flow. Taken together, it is reasonable to hypothesize that perfusion flow mitigates hypoxic conditions by providing adequate oxygen and nutrient delivery to the center of 3D constructs. Thus, although VEGF is up-regulated in differentiated osteoblasts, the reduction in VEGF with perfusion flow may provide evidence that the role of VEGF in hypoxia may supersede its role in osteoblast differentiation.

The increase in endothelial cell aggregation length highlights the potential for perfusion bioreactors in 3D bone tissue engineering. By increasing the *in vitro* activity of ECs in bone tissue constructs, perfusion flow could serve to speed up the process of host acceptance in future clinical applications. A higher quality vascular-like network *in vitro* will have a greater likelihood of attaining successful anastomosis with host vasculature *in vivo*. Even if functional anastomosis isn't realized, perfusion-induced EC activity could potentiate paracrine signaling between cells within the scaffold and host vasculature, advancing vascular infiltration.

Our results support the continued use of perfusion bioreactors to culture 3D bone tissue constructs. Perfusion flow increased osteoblast mRNA production, cell number, and cell coverage area. Furthermore, perfusion flow stimulated an increase in endothelial cell aggregation length in osteoblast/endothelial cell co-cultures. These results support the use of perfusion bioreactors to improve *in vitro* co-culturing in order to maximize the quality and effectiveness of vascularized bone tissue cultures.

Acknowledgments

Support from the National Science Foundation Graduate Research Fellowship Program and the Michigan Space Grant Consortium.

References

1. Yaszemski MJ, Payne RG, Hayes WC, Langer R, Mikos AG 1996 Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. Biomaterials **17**(2)**:**175-85.

2. Bauer TW 2007 An overview of the histology of skeletal substitute materials. Arch Pathol Lab Med **131**(2)**:**217-24.

3. Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA 2007 Cell-Based Bone Tissue Engineering. PLoS Med **4**(2)**:**e9.

4. Muschler GF, Nakamoto C, Griffith LG 2004 Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am **86-A**(7)**:**1541-58.

5. Langer R, Vacanti JP, Vacanti CA, Atala A, Freed LE, Vunjak-Novakovic G 1995 Tissue engineering: biomedical applications. Tissue Eng **1**(2)**:**151-61.

6. Galletti PM, Hellman KB, Nerem RM 1995 Tissue engineering: from basic science to products: a preface. Tissue Eng **1**(2)**:**147-9.

7. Choong CS, Hutmacher DW, Triffitt JT 2006 Co-culture of Bone Marrow Fibroblasts and Endothelial Cells on Modified Polycaprolactone Substrates for Enhanced Potentials in Bone Tissue Engineering. Tissue Eng.

8. Stahl A, Wenger A, Weber H, Stark GB, Augustin HG, Finkenzeller G 2004 Bi-directional cell contact-dependent regulation of gene expression between endothelial cells and osteoblasts in a three-dimensional spheroidal coculture model. Biochem Biophys Res Commun **322**(2)**:**684-92.

9. Wenger A, Kowalewski N, Stahl A, Mehlhorn AT, Schmal H, Stark GB, Finkenzeller G 2005 Development and characterization of a spheroidal coculture model of endothelial cells and fibroblasts for improving angiogenesis in tissue engineering. Cells Tissues Organs **181**(2)**:**80-8.

10. Unger RE, Sartoris A, Peters K, Motta A, Migliaresi C, Kunkel M, Bulnheim U, Rychly J, Kirkpatrick CJ 2007 Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials. Biomaterials **28**(27)**:**3965-76.

11. Kanczler JM, Oreffo RO 2008 Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater **15:**100-14.

12. Carano RA, Filvaroff EH 2003 Angiogenesis and bone repair. Drug Discov Today **8**(21)**:**980-9.

13. Glowacki J 1998 Angiogenesis in fracture repair. Clin Orthop Relat Res (355 Suppl)**:**S82-9.

14. John P. Bilezikian LGR, Gideon A. Rodan 2002 Principles of Bone Biology. In: Bilezikian R, Rodan (ed.) Principles of Bone Biology, 2nd ed., vol. 1 and 2. Academic Press, San Diego, pp 1700.

15. Burkhardt R, Kettner G, Bohm W, Schmidmeier M, Schlag R, Frisch B, Mallmann B, Eisenmenger W, Gilg T 1987 Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. Bone **8**(3)**:**157-64.

16. Hausman MR, Schaffler MB, Majeska RJ 2001 Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone **29**(6)**:**560-4.

17. Rouwkema J, de Boer J, Van Blitterswijk CA 2006 Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. Tissue Eng **12**(9)**:**2685-93.

18. Villars F, Guillotin B, Amedee T, Dutoya S, Bordenave L, Bareille R, Amedee J 2002 Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. Am J Physiol Cell Physiol **282**(4)**:**C775-85.

19. Akita S, Tamai N, Myoui A, Nishikawa M, Kaito T, Takaoka K, Yoshikawa H 2004 Capillary vessel network integration by inserting a vascular pedicle enhances bone formation in tissue-engineered bone using interconnected porous hydroxyapatite ceramics. Tissue Eng **10**(5-6)**:**789-95.

20. Villars F, Bordenave L, Bareille R, Amedee J 2000 Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? J Cell Biochem **79**(4)**:**672-85.

21. Clarkin CE, Emery RJ, Pitsillides AA, Wheeler-Jones CP 2008 Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated crosstalk to endothelial cells. J Cell Physiol **214**(2)**:**537-44.

22. Steffens L, Wenger A, Stark GB, Finkenzeller G 2009 In vivo engineering of a human vasculature for bone tissue engineering applications. J Cell Mol Med **13**(9B)**:**3380-6.

23. Yu H, VandeVord PJ, Mao L, Matthew HW, Wooley PH, Yang SY 2009 Improved tissue-engineered bone regeneration by endothelial cell mediated vascularization. Biomaterials **30**(4)**:**508-17.

24. Santos MI, Reis RL Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. Macromol Biosci **10**(1)**:**12-27.

25. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE 2003 Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng **9**(6)**:**1197-203.

26. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG 2002 Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A **99**(20)**:**12600-5.

27. Jaasma MJ, O'Brien FJ 2008 Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A **14**(7)**:**1213-23.

28. Du D, Furukawa KS, Ushida T 2009 3D culture of osteoblast-like cells by unidirectional or oscillatory flow for bone tissue engineering. Biotechnol Bioeng **102**(6)**:**1670-8.

29. Li D, Tang T, Lu J, Dai K 2009 Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. Tissue Eng Part A **15**(10)**:**2773-83.

30. Volkmer E, Drosse I, Otto S, Stangelmayer A, Stengele M, Kallukalam BC, Mutschler W, Schieker M 2008 Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. Tissue Eng Part A **14**(8)**:**1331-40.

31. Holtorf HL, Jansen JA, Mikos AG 2006 Modulation of cell differentiation in bone tissue engineering constructs cultured in a bioreactor. Adv Exp Med Biol **585:**225-41.

32. Vance J, Galley S, Liu DF, Donahue SW 2005 Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release. Tissue Eng **11**(11-12)**:**1832-9.

33. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG 2003 Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci U S A **100**(25)**:**14683-8.

34. Wang Y, Uemura T, Dong J, Kojima H, Tanaka J, Tateishi T 2003 Application of perfusion culture system improves in vitro and in vivo osteogenesis of bone marrow-derived osteoblastic cells in porous ceramic materials. Tissue Eng **9**(6)**:**1205-14.

35. Plunkett NA, Partap S, O'Brien FJ Osteoblast response to rest periods during bioreactor culture of collagen-glycosaminoglycan scaffolds. Tissue Eng Part A **16**(3)**:**943-51.

36. Zhao F, Chella R, Ma T 2007 Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: Experiments and hydrodynamic modeling. Biotechnol Bioeng **96**(3)**:**584-95.

37. Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG 2006 In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci U S A **103**(8)**:**2488-93.

38. Holtorf HL, Datta N, Jansen JA, Mikos AG 2005 Scaffold mesh size affects the osteoblastic differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor. J Biomed Mater Res A **74**(2)**:**171-80.

39. Street J, Bao M, deGuzman L, Bunting S, Peale FV, Jr., Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van Bruggen N, Redmond HP, Carano RA, Filvaroff EH 2002 Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci U S A **99**(15)**:**9656-61.

40. Ozawa N, Shichiri M, Iwashina M, Fukai N, Yoshimoto T, Hirata Y 2004 Laminar shear stress up-regulates inducible nitric oxide synthase in the endothelium. Hypertens Res **27**(2)**:**93-9.

41. Yamamoto K, Takahashi T, Asahara T, Ohura N, Sokabe T, Kamiya A, Ando J 2003 Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. J Appl Physiol **95**(5)**:**2081-8.

42. Fidkowski C, Kaazempur-Mofrad MR, Borenstein J, Vacanti JP, Langer R, Wang Y 2005 Endothelialized microvasculature based on a biodegradable elastomer. Tissue Eng **11**(1-2)**:**302-9.

43. Cowin SC 2001 Bone mechanics handbook, 2nd ed. CRC Press, Boca Raton, FL, pp 1 v. (various pagings).

44. Cornell CN 1999 Osteoconductive materials and their role as substitutes for autogenous bone grafts. Orthop Clin North Am **30**(4)**:**591-8.

45. Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29**(9)**:**e45.

46. Gomes ME, Sikavitsas VI, Behravesh E, Reis RL, Mikos AG 2003 Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. J Biomed Mater Res A **67**(1)**:**87-95.

47. Malda J, Klein TJ, Upton Z 2007 The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng **13**(9)**:**2153-62.

48. Griffith CK, Miller C, Sainson RC, Calvert JW, Jeon NL, Hughes CC, George SC 2005 Diffusion limits of an in vitro thick prevascularized tissue. Tissue Eng **11**(1-2)**:**257-66.

49. Meury T, Verrier S, Alini M 2006 Human endothelial cells inhibit BMSC differentiation into mature osteoblasts in vitro by interfering with osterix expression. J Cell Biochem **98**(4)**:**992-1006.

50. Potier E, Ferreira E, Andriamanalijaona R, Pujol JP, Oudina K, Logeart-Avramoglou D, Petite H 2007 Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone **40**(4)**:**1078-87.

51. Steinbrech DS, Mehrara BJ, Saadeh PB, Chin G, Dudziak ME, Gerrets RP, Gittes GK, Longaker MT 1999 Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro. Plast Reconstr Surg **104**(3)**:**738-47.

CHAPTER SEVEN
Conclusions

Summary

3D bone tissue-engineering is constrained both in vitro and in vivo by limited cellular viability. In vitro, large 3D structures experience reduced osteogenic activity in their centers due to diffusional restrictions (1-10). In vivo, 3D tissue-engineered constructs are faced with limited vascular infiltration after implantation, resulting in cellular apoptosis (11,12). While perfusion bioreactors show promise for increasing cellular viability and osteogenic activity in vitro (1-8), it remains unclear how mechanical stimulation contributes to long-term 3D cultures. In addition to improving culture conditions for 3D structures in vitro (Chapters 4 and 6), perfusion flow also increased the length of endothelial cell aggregations (Chapter 6), which could lead to improved vascular integration upon in vivo implantation.

Though partitioning mechanical stimulation did not establish an increase in osteogenic response, initial experimentation did provide significant, although unexpected, results. OPN gene expression was reduced when osteoblasts cultured on 3D calcium phosphate scaffolds were exposed to high-shear oscillatory fluid flow for 24 hours (Chapter 3), which contrasts 2D results. Furthermore, OPN, VEGF, and HIF-1α gene expression was reduced when osteoblasts cultured on 3D scaffolds were exposed to a constant rate of low-shear perfusion flow (24 hours for OPN and VEGF, 48 hours for HIF-1α) (Chapter 4). Literature suggests that OPN gene expression is increased by mechanical stimulation (7,8,13-16), but there is also evidence that OPN is increased under hypoxic conditions (17-19). I believe that in our model, OPN's role in hypoxia may supersede its response to mechanical stimulation. Supporting evidence includes the reduction in both VEGF and HIF-1α (major mediators that are increased with hypoxia), as well as increases in osteoblast infiltration (Chapter 4), cell number, scaffold perimeter coverage, and cell area coverage in osteoblasts cultured with perfusion flow (Chapter 6). Future work must consider the extent of induced hypoxia in our 3D constructs.

Increased vascular-like structure in 3D in vitro scaffolds may accelerate construct integration upon in vivo implantation. As perfusion flow increased the length of endothelial cell aggregations, the use of perfusion bioreactors to improve vascular-like networks in co-cultures is promising. Future studies are necessary to better characterize a co-culture model that could maximize matrix deposition as well as vascular network formation.

Key Findings

- Partitioning mechanical stimulation does not alter gene expression in comparison to a single bout.
- Gene expression of OPN and FGF-2 is up-regulated in 3D compared to 2D, while Collagen 1 gene expression is down-regulated.
- Mechanical stimulation increases OPN in 2D culture at 48 hours, but decreases OPN in 3D culture.
- Perfusion flow decreases hypoxia-induced genes: VEGF, HIF-1α, OPN.
- ALP is up-regulated, and OCN and down-regulated in 2D osteoblast/endothelial cell co-cultures. 3D co-cultures did not provide similar results.
- ALP and OCN gene expression is up-regulated in long-term osteoblast cultures exposed to perfusion flow.
- VEGF expression is reduced with perfusion flow in long-term osteoblast cultures.
- Cell number, scaffold perimeter coverage, and cell area coverage are all increased in the center of 3D scaffolds cultured long-term, but are not increased around the scaffold perimeter.

Limitations

One of the biggest limitations of this dissertation is that many of the conclusions are drawn from gene expression data. While this data is critical to fully understanding signal transduction in 3D cultures, transcription does not necessarily guarantee translation. Thus, while gene expression may be altered, protein production may not change. However, the final study (see Chapter 6) included histological evidence that

supports many of the conclusions drawn from gene expression data. None-the-less, it would be of interest to establish gene and protein expression along with matrix production and calcium deposition to chronicle patterns of bone growth in a 3D in vitro environment.

One recognized limitation of our study involving 2D vs. 3D is the disparity in substrates between models. With glass as the substrate in our 2D model, and calcium phosphate as the porous scaffold in our 3D model, it is possible that some of the changes in gene expression are due to differences in material characteristics, rather than architecture as we believe. To truly understand the differences that material properties have on gene expression, it will be necessary to design experiments controlling for architecture.

In Chapter 6, we sought to test the effects of co-culture on gene expression in our 3D model. Our results suggested that co-culturing decreases OPN and RUNX-2 gene expression in osteoblasts cultured in 3D as well as OCN gene expression in 2D (results not shown), suggesting that co-culture may indeed slow the process of differentiation in osteoblasts. However, there is no histological evidence of changes in OB activity, as cell perimeter, area, and number were not significantly different and showed no distinct trends between co-cultures and osteoblasts alone. Nevertheless, these histological measures may not be the best indication of osteoblast differentiation. Unfortunately, one of the best measures of osteoblast maturation is the deposition of calcium, which is best measured analytically. As our scaffolds consist of calcium phosphate, any changes in calcium deposition would be extremely difficult to measure. Furthermore, it was not possible to measure calcium deposition histologically, because we were unable to determine the difference between the scaffold and fresh calcium deposits.

While it was exciting to show an increase in endothelial aggregation length, we were not able to provide evidence of the formation of vascular-like networks among endothelial cell populations. It is possible that rather than migrating together (as has been shown in literature), endothelial cells proliferated in “nodes” to align themselves along the perimeter of the scaffolds’ pores. This method of formation seems likely as we did not observe any network formation between aggregations, nor did we identify any individual endothelial cells outside of major aggregations. Furthermore, we found no

evidence of the formation of a vessel lumen within aggregations, diminishing the hope for functional anastomosis with native bone. However, even if anastomosis upon implantation is not achieved, the incorporation of EC's into 3D cultures may still improve vascularization through paracrine signaling activity between cells within the scaffold and host vasculature.

Future Direction

Mechanical Stimulation in 2D and 3D

It is clear that differences do exist between 2D and 3D cultures, suggesting that data collected in 2D studies cannot necessarily be extrapolated to a 3D environment. Furthermore, mechanical stimulation can have different effects in 2D vs. 3D, highlighting the importance of future experimentation on mechanotransduction in 3D culture. Responses to mechanical stimulation may be partially driven by factors such as architecture, which cannot be completely accounted for in 2D cultures. Future research must focus on controlling the parameters of a 3D grafting structure, and comparing 2D and 3D cultures under dynamic conditions.

The Role of OPN

Mechanical stimulation and perfusion flow both reduced the gene expression of OPN, which is generally thought to be a marker of osteoblast differentiation in long-term 3D cultures. As such, it was surprising that early time points showed a significant decrease in its expression. Knowing that OPN also plays a major role in the inflammatory response as well as a role in hypoxia, it would be prudent to study OPN more carefully, including a time course to elucidate its role in 3D cultures. Considering our results, it is possible that different mechanisms may drive OPN gene expression in 3D cultures.

Vascular Network Formation in 3D Co-cultures

The formation of a vascular network was not achieved in 3D osteoblast/endothelial cell co-cultures. As stated earlier, it is more likely that ECs

proliferated and continued to align themselves along the perimeter of the scaffolds' pores to form aggregates, rather than actively migrating together as is seen in other models. The low ratio (2%) of endothelial cell seeding may not have allowed for communication between populations of endothelial cells, which is necessary for network formation. In the future, it may be necessary to increase the ratio of endothelial cells to increase interaction between aggregations, enhancing network formation.

Another potential reason for the lack of network formation is the cell type. While the EOMA cell line has provided a good benchmark, primary cell lines may prove to form more mature vascular-like networks.

Vascular Anastomosis

Ideally, a vascularized tissue-engineered bone construct would enhance anastomosis between the vascular network provided by the 3D implant and the host's vascular supply, forming functional connections. While this has seen limited success in vivo, future research should include in vivo implantations of vascularized constructs enhanced by perfusion flow. Even if functional anastomosis isn't realized, perfusion-induced endothelial cell activity may paracrine signaling between cells within the scaffold and host vasculature, advancing vascular infiltration despite a lack of a functional association.

Hypoxia

Evidence supports the hypothesis that perfusion flow reduces hypoxic conditions in 3D tissue-engineered scaffolds. Down-regulation of hypoxia-specific genes, as well as increases in cell number, perimeter coverage, and cell area coverage in the center of 3D scaffolds all suggest that perfusion flow provides delivery of oxygen and nutrients to starved cells. However, future research must directly measure whether hypoxia exists in the center of in vitro scaffolds. Existing literature suggests that 3D structures do indeed experience hypoxia, but our model must be tested to determine whether hypoxic conditions exist, and the extent of rescue that perfusion flow provides.

References

1. Vance J, Galley S, Liu DF, Donahue SW 2005 Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release. Tissue Eng 11(11-12):1832-9.

2. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE 2003 Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng 9(6):1197-203.

3. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG 2002 Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A 99(20):12600-5.

4. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG 2003 Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci U S A 100(25):14683-8.

5. Gomes ME, Sikavitsas VI, Behravesh E, Reis RL, Mikos AG 2003 Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. J Biomed Mater Res A 67(1):87-95.

6. Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG 2006 In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci U S A 103(8):2488-93.

7. Jaasma MJ, O'Brien FJ 2008 Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A 14(7):1213-23.

8. Plunkett NA, Partap S, O'Brien FJ Osteoblast response to rest periods during bioreactor culture of collagen-glycosaminoglycan scaffolds. Tissue Eng Part A 16(3):943-51.

9. Malda J, Klein TJ, Upton Z 2007 The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng 13(9):2153-62.

10. Griffith CK, Miller C, Sainson RC, Calvert JW, Jeon NL, Hughes CC, George SC 2005 Diffusion limits of an in vitro thick prevascularized tissue. Tissue Eng 11(1-2):257-66.

11. Santos MI, Reis RL Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. Macromol Biosci 10(1):12-27.

12. Kanczler JM, Oreffo RO 2008 Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater 15:100-14.

13. You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR 2001 Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem 276(16):13365-71.

14. Ponik SM, Triplett JW, Pavalko FM 2007 Osteoblasts and osteocytes respond differently to oscillatory and unidirectional fluid flow profiles. J Cell Biochem 100(3):794-807.

15. Wu CC, Li YS, Haga JH, Wang N, Lian IY, Su FC, Usami S, Chien S 2006 Roles of MAP kinases in the regulation of bone matrix gene expressions in human osteoblasts by oscillatory fluid flow. J Cell Biochem 98(3):632-41.

16. Batra NN, Li YJ, Yellowley CE, You L, Malone AM, Kim CH, Jacobs CR 2005 Effects of short-term recovery periods on fluid-induced signaling in osteoblastic cells. J Biomech 38(9):1909-17.

17. Potier E, Ferreira E, Andriamanalijaona R, Pujol JP, Oudina K, Logeart-Avramoglou D, Petite H 2007 Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone 40(4):1078-87.

18. Raheja LF, Genetos DC, Yellowley CE 2008 Hypoxic osteocytes recruit human MSCs through an OPN/CD44-mediated pathway. Biochem Biophys Res Commun 366(4):1061-6.

19. Gross TS, King KA, Rabaia NA, Pathare P, Srinivasan S 2005 Upregulation of osteopontin by osteocytes deprived of mechanical loading or oxygen. J Bone Miner Res 20(2):250-6.

Appendix A

COPYRIGHT AGREEMENT (as of December 2008)

ASME Publishing • Three Park Avenue • New York, NY 10016 ASME prefers that you return this form to: copyright@asme.org or fax the form to: FAX 212-591-7060 or 212-591-7292

Before publication of your paper in a conference proceeding or in a journal, ASME must receive your signed Copyright Agreement Form. For conference papers, this form should be received by the deadline indicated by the Conference. Other forms may NOT be substituted for this form, nor may any wording on the form be changed. HANDWRITTEN SIGNATURES ONLY are acceptable.

Please fill in the information requested below. The copyright owner(s) of the Paper should then sign this form as instructed below. Please refer to our FAQ page for more information regarding copyright ownership.

PAPER NUMBER (for conference papers): BIO-09-1357
Multiple forms may be submitted.

TITLE: Mechanical Stimulation Mediates Gene Expression in MC3T3 Osteoblastic Cells Differently in 2D & 3D Environments

AUTHOR(s): Matthew J. Barron, B.S. (Lead Author), Chung-Jui Tsai, Ph.D., Seth W. Donahue, Ph.D. (designated author)

CONFERENCE NAME: _____

JOURNAL NAME: Biomechanical Engineering

COPYRIGHT ASSIGNMENT

The undersigned hereby assigns irrevocably to ASME all worldwide rights under copyright in the Paper.

Authors retain all proprietary rights in any idea, process, procedure, or articles of manufacture described in the Paper, including the right to seek patent protection for them. Authors may perform, lecture, teach, conduct related research and display all or part of the Paper, in print or electronic format. Authors may reproduce and distribute the Paper for non-commercial purposes only. For all copies of the Paper made by Authors, Authors must acknowledge ASME as original publisher and include the names of all author(s), the publication title, and an appropriate copyright notice that identifies ASME as the copyright holder.

PLEASE READ THE TERMS AND CONDITIONS WHICH ARE FULLY INCORPORATED IN THIS AGREEMENT.

Who Should Sign:

Only the copyright owner(s) of the Paper can sign below. If one of the following applies you may not own the copyright of the paper and may need to have the appropriate copyright owner(s) sign this Agreement:
(1) you created the Paper within the scope of your employment, and your employer is the copyright owner;
(2) you created the Paper under an independent contractor agreement**; or
(3) you received a grant that funded your Paper.
Please review applicable company, institutional, and grant policies and your employment/independent contractor agreement to determine who holds the rights to your Paper. For more information, please refer to the FAQs

****Note to U.S. Government Contractors**: If you created the Paper under contract with the U.S. Government, it is not necessarily a public domain work. You or your employer may own the copyright. Please review your company/institutional policies and your contractor agreement. Your Paper may require a footer acknowledging contract information and also the following statement:

"The United States Government retains, and by accepting the article for publication, the publisher acknowledges that the United States Government retains, a non-exclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this work, or allow others to do so, for United States Government purposes."

Please be sure that the final PDF version of the Paper you submit includes all necessary footers and statements required under your contract.